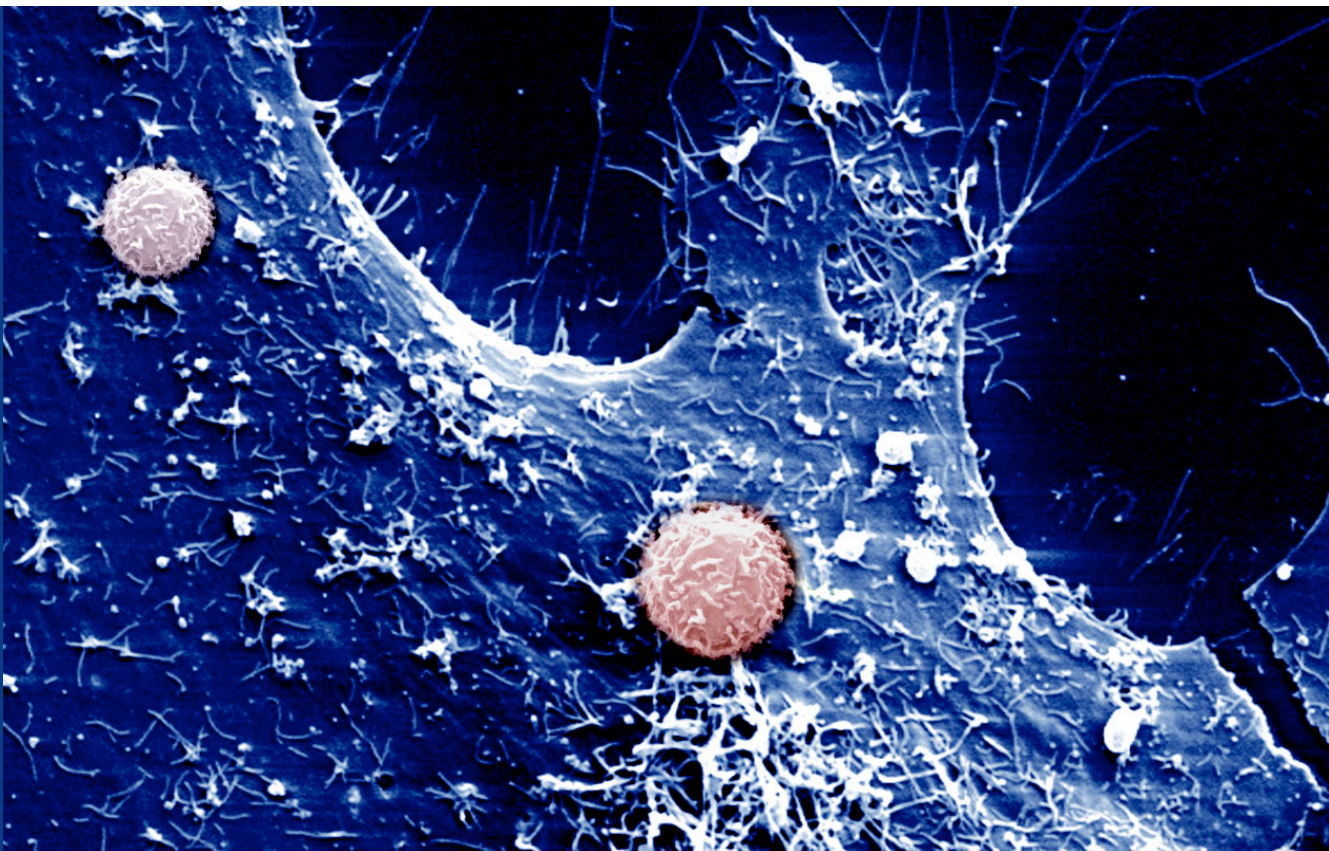
HELSINGIN YLIOPISTO
HELSINGFORS UNIVERSITET
UNIVERSITY OF HELSINKI

Target Cell Topography and Cytoskeletal Reorganization in Natural Killer Cell Activity

Tuula S. Helander



Department of Pathology
Haartman Institute
Faculty of Medicine
University of Helsinki
Finland

Target Cell Topography and Cytoskeletal Reorganization in Natural Killer Cell Activity

Tuula S. Helander

ACADEMIC DISSERTATION

To be presented and publicly discussed with the permission of the Faculty of Medicine, University of Helsinki, in the Small Lecture Hall of the Haartman Institute, Haartmaninkatu 3, on March 26th, 2010, at 12 o´clock noon.

Helsinki 2010

Supervised by

Docent Tuomo Timonen, M.D., Ph.D.
Meilahti Laboratories of Pathology, HUSLAB
Helsinki University Central Hospital

Department of Pathology, Haartman Institute
University of Helsinki
Helsinki, Finland

Reviewed by

Docent Sampsa Matikainen, Ph.D.
Unit of Excellence for Immunotoxicology
Finnish Institute of Occupational Health
Helsinki, Finland

and

Professor Marko Salmi, M.D., Ph.D.
MediCity Research Laboratory
University of Turku
Turku, Finland

Opponent

Professor Olli Silvennoinen, M.D., Ph.D.
Institute of Medical Technology
University of Tampere
Tampere, Finland

Front Cover Illustration: Two NK cells adhering to a target cell expressing early viral protein (nsP1) that sensitizes the target cell to NK cell killing (SEM photo by Pekka Kujala, and modified by Tuula Helander (manuscript).

ISBN 978-952-92-6884-9 (paperback)
ISBN 978-952-10-6097-7 (PDF)
http://ethesis.helsinki.fi

Helsinki University Print
Helsinki 2010

"Express Yourself or Die"
Kärre K., Science 1995

Contents

List of Original Publications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-III). The original publications have been reprinted with the kind permission of the copyright holders. Some unpublished data have also been included in the thesis.

I **Helander, T.,** Timonen, T., Kalliomäki, P. & Schröder, J. 1991. Recognition of chromosome 6-associated target structures by human lymphokine-activated killer cells. *J. Immunol.* 147: 2063-2067.

II **Helander, T.S.,** Carpén, O., Turunen, O., Kovanen, P.E., Vaheri, A. & Timonen, T. 1996. ICAM-2 redistributed by ezrin as a target for killer cells. *Nature*, 382: 265-268.

III **Helander, T.S.,** Auvinen, P., Kujala, P, Vaheri, A., Kääriäinen, L. & Timonen T. Viral early protein nsP1 sensitizes target cells to NK killing – Role of cellular extensions containing colocalized ezrin and nsP1. *Manuscript.*

Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
APC	antigen presenting cell
CD	cluster of differentiation
CISS	chromosomal *in situ* suppression hybridization
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DC	dendritic cell
ERM	ezrin - radixin - moesin
F-actin	filamentous actin
FITC	fluorescein isothiocyanate
HA	influenza virus hemagglutinin
HLA	human leukocyte antigen
HSV	Herpes Simplex Virus
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ITAM	immunoreceptor tyrosine-based activatory motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
KIR	killer cell immunoglobulin-like receptor
LAK	lymphokine activated killer
LFA	lymphocyte function-associated antigen
LGL	large granular lymphocyte
mAb	monoclonal antibody
MHC	major histocompatibility complex
MICA, B	major histocompatibility complex class I chain-related A and B chain

NCR	natural cytotoxicity receptor
NK	natural killer
nsP	nonstructural protein(s)
PBL	peripheral blood lymphocyte(s)
p.i.	post infection
SFV	Semliki Forest Virus
SEM	scanning electron microscopy
TCR	T cell receptor
TEM	transmission electron microscopy
Th	helper T lymphocyte
TNF	tumor necrosis factor
TRITC	tetramethyl rhodamine isothiocyanate
Ts	suppressor T lymphocyte
wt	wild type

Abstract

Cell-mediated cytotoxicity is a highly organized multifactorial process inducing target cell death through cytotoxic effector cells. Its most important function is to remove abnormal or infected cells in order to prevent the development of malignancies and to eliminate intracellular pathogens.

Natural killer (NK) cells directly recognize and kill transformed or virus-infected cells without prior sensitization. NK cell functions, cytotoxicity and cytokine production, are regulated by a balance between negative and positive signals initiated after the engagement of cell surface inhibitory and activating receptors that bind to specific ligands on altered and normal cells.

We have studied both virus-infected and tumor cells in order to identify the target structures involved in triggering NK activity.

To investigate the target cell sensitivity and the structures involved in NK cell activity, mouse/human cell hybrids containing various human chromosomes were used as targets. The human chromosome responsible for activating NK cell killing was identified to chromosome number 6. The chromosome 6-hybrid cells were resistant to non-activated NK cells but were very efficiently bound to and killed by interleukin 2-activated (IL-2) killer cells. The hybrids containing several other human chromosomes, but not number 6, were not sensitive to any killer activity. The results suggest that activated NK cells recognize ligands that are encoded on human chromosome 6.

The binding and cytotoxicity of NK sensitive human chromosome 6-hybrids were effectively inhibited by antibodies against lymphocyte function-associated antigen, LFA-1 (adhesion receptor on effector cells). We showed that the ligand on the target cell side was intercellular adhesion molecule 2 (ICAM-2). There was no difference in the level of expression of ICAM-2, however, but a drastic difference was seen in the distribution of the molecule: ICAM-2 was evenly distributed on the surface of the NK-resistant cells, but almost totally redistributed to the tip of uropods, bud-like extensions, which were absent from the parental

cells. ICAM-1, another ligand of the LFA-1 molecule, was evenly distributed in both cell lines.

The results demonstrate that human chromosome 6 induces accumulation of ICAM-2 onto the tips of uropods present in NK cell-sensitive hybrids. Interestingly, the gene coding for the cytoskeletal linker protein ezrin has been localized to the long arm of human chromosome 6. The results of both immunoblotting and immunofluorescence assays showed that 6-hybrids expressed both mouse and human ezrin, and there was a colocalization of ezrin and ICAM-2 in the uropods. Furthermore, the transfected human ezrin into NK cell-resistant cells induced uropod formation, ICAM-2 and ezrin redistribution to newly formed uropods, and sensitized target cells to NK cell killing.

The results shed light on a novel mechanism of target cell recognition by killer cells: target structures are normal structures, already present on the surface of normal (healthy) cells, but in pathological conditions structures are concentrated into biologically active foci due to aberrant cytoskeletal interactions.

NK cells are central players in the defence against virus infections by the secretion of antiviral cytokines, such as interferon gamma (IFN-γ), or by the direct lysis of virus-infected cells. They inhibit the spread of infection, allowing time for specific immune responses to develop. Surprisingly little data are available on the direct cytotoxicity of virus-infected targets by NK cells.

Semliki Forest virus (SFV) was used as a model to study the viral structures that could be central in NK activity. The SFV-specific non-structural proteins (nsP1-4) translated early in the virus cycle were transfected in NK-resistant cells using vaccinia virus expression vector, which allows efficient and rapid synthesis of plasmid-encoded proteins. Viral gene nsP1 alone efficiently sensitized target cells to NK activity. NsP1 localized to the cytoplasmic surface of the viral induced filopodia-like extensions. The tight membrane association of nsP1 seems to be critical in the triggering of NK killing, since the nsP1-mutant devoid of membrane-binding sequences did not sensitize transfectants to NK activity. It seems that NK cells recognize virus-infected cells at a very early stage, long before any viral structural proteins are produced.

The present SFV-model, which shows that structural changes are associated with NK sensitivity, suggests that in viral infections NK cells react to rapid changes in membrane topography, and thus facilitate the generation of a more efficient T cell system through cytotoxicity and cytokine production.

Review of the Literature

Introduction

The immune system has to recognize and destroy abnormal or infected cells to maintain homeostasis. The immune response is traditionally divided into two broad systems which provide innate and adaptive immunity. These systems ensure protection to potentially pathogenic microorganisms, such as viruses, bacteria, fungi and parasites. If infection occurs, usually the pathogen is rapidly recognized and destroyed, and long lasting immunity ensues. An adaptive immune response provides specific immunity, but the first line defence against pathogens is the innate system. Natural killer (NK) cells comprise a unique subset of immune cells that have long been considered as innate immune cells.

Tumors are formed by cells that have overcome normal growth regulation mechanisms. Therefore the ability to distinguish normal, healthy cells from abnormal ones is a key element of selectively attacking tumor cells. NK cells have various receptor systems designed to recognize abnormal cells. Besides killing the altered cells, the target cell recognition can also induce NK cells to secrete several cytokines and chemokines thus regulating both innate and adaptive immune response (Cooper et al., 2001).

1. Cytotoxic cells of the immune system

Many unique cell types respond to pathogen-derived molecules and play key roles in combating infections or otherwise deranged cells. Innate immune cells, such as macrophages and dendritic cells (DC), directly kill the pathogenic micro-organisms through phagocytosis, or produce cytokines that aid elimination of the

pathogens and instruct adaptive immune response (Akira et al., 2006; Medzhitov, 2007). Immunity against the pathogens depends on the recognition of pathogen-derived structures in the plasma membrane or in the cytosol by receptors that are collectively known as pattern-recognition receptors (PRR). Of these, the Toll-like receptors (TLR) are best characterized (Diacovich and Gorvel, 2010). These innate receptors are evolutionary conserved and are encoded in the germline of multicellular organisms (including insects and jawed vertebrates). TLR are broadly expressed on leukocytes, and some receptors are also expressed on human NK cells (e.g. TLR1, TLR6 and TLR9) suggesting that they may also participate in triggering immune responses (Hornung et al., 2002).

The other group, adaptive immune cells, such as T and B lymphocytes, react with specificity to peptides derived from pathogens, expand clonally, and usually after a week of infection the immune response takes place. T cells can be roughly divided into two main classes: cytotoxic T cells (CTL) kill virus-infected cells, and helper T cells (Th) activate other innate and adaptive immune cells. B cells, when activated and differentiated into plasma cells, secrete pathogen-specific antibodies. Despite many similarities between the innate and adaptive immune response (activation, proliferation, cytokine production), the critical distinction is the formation of immunologic memory by the adaptive immune cells.

The cytotoxic activity is efficiently performed by NK cells and CTL. Regardless of acting upon target cells in the same way – inducing programmed cell death, called apoptosis – the mechanisms of lymphocyte-mediated cell recognition are different. CTL kill target cells by processing and presenting specific antigens through the major histocompatibility complex (MHC) class I molecules. In contrast, NK cells destroy target cells in which the expression of MHC is reduced or deficient. Thus, NK cells can recognize alterations that cannot be detected by the peptide/MHC-specific T cells. NK cells detect loss of expression of self-MHC protein: this concept of recognition is known as “missing self hypothesis” (*Fig. 1*) (Ljunggren and Karre, 1985; Ljunggren and Karre, 1990; Karre, 1995; Karre, 2008). Although human red blood cells do not express MHC class I molecules, they are not killed by NK cells, suggesting that erythrocytes lack NK activating ligands.

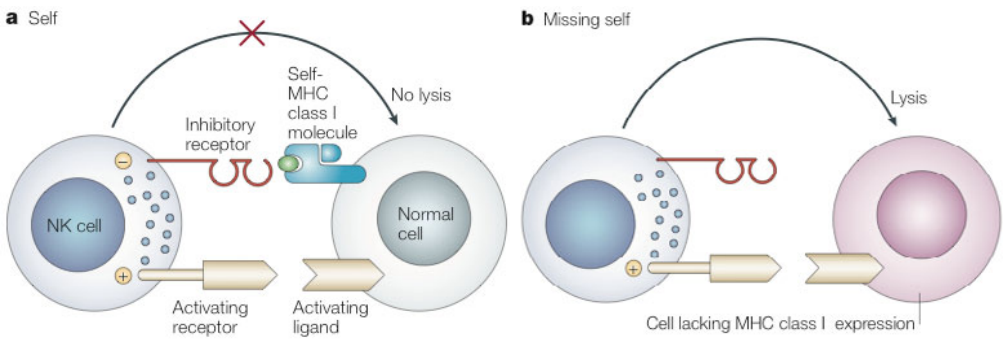


Figure 1. "Missing-self" hypothesis. An NK cell does not lyse the healthy (autologous) target cell that expresses the appropriate self-MHC class I alleles which are recognized by inhibitory receptors (A). An NK cell eliminates the target cell because the critical "self" proteins are absent from the target cell surface (e.g. due to viral infection or transformation) (B). Modified from Kumar & McNerney (2005).

During virus infection, T cells can recognize viral peptides presented on MHC class I leading to elimination of virus-infected cells. However, some viruses (such as cytomegalovirus, CMV) inhibit all host protein synthesis, and then also the MHC class I protein synthesis is blocked (Lanier, 2008). Thus the NK cells are no longer inhibited through their MHC-specific receptors, but are ready to activate their cytolytic machinery. Some viruses (such as HIV) are also able to prevent export of MHC class I molecules, which allows the infected cells to evade recognition by CTL, although they are still sensitive for NK cell killing (Swann et al., 2001).

According to the "missing self" hypothesis, NK cells recognize and eliminate cells that fail to express self MHC class I molecules. However, there are also NK-sensitive, malignant cells that express MHC class I molecules, as well as NK-resistant tumor cells with diminished expression of MHC I (Garcia-Lora et al., 2003; Bubenik, 2004; Aptsiauri et al., 2007).

2. Brief overview of NK cells

NK cells were first characterized more than 35 years ago. Their discovery was based on their ability to "spontaneously" kill certain tumor cells (Herberman et al., 1975; Kiessling et al., 1975; Kiessling and Wigzell, 1979). They serve in the first line of defence against a variety of infections. This is best demonstrated by a

patient who had complete and selective NK cell deficiency, and suffered from continuous, severe herpes virus infections that were eventually fatal (Biron et al., 1989; Lodoen and Lanier, 2006; Bustamante et al., 2008). NK cells are mainly generated in bone marrow, although other organs such as spleen, fetal liver, lymph nodes and thymus have been proposed to engage in NK cell development (Freud and Caligiuri, 2006; Huntington et al., 2007).

2.1 Morphology

Morphologically NK cells are large granular lymphocytes (LGL) (Timonen et al., 1981). Their azurophilic granules are rich in cytolytic enzymes, such as perforin and granzymes (Timonen et al., 1981; Trinchieri, 1989). Also some activated CTL may have LGL morphology, but NK cells are clearly not T cells even though they sometimes share some effector functions and surface molecules (Lanier et al., 1986c).

2.2 Surface markers

Human NK cells express several markers associated also with other cells, but they closely resemble T cells. They express CD56, which is also found on a fraction of T cells, but they do not express the T-cell receptor complex (TCR/CD3) nor do they rearrange the TCR genes (Lanier et al., 1986a).

CD56 is a molecule derived from alternative splicing of the gene encoding the neural cell adhesion molecule (NCAM) involved in nervous system development and cell-cell interactions (Hercend et al., 1985; Lanier et al., 1986b; Cunningham et al., 1987; Rutishauser et al., 1988; Lanier et al., 1989b). NK cells do not express the CD3 components, typical for T cells, except for the CD3 zeta chain, which is associated with CD16 (FcγRIII) and other NK cell activation receptors (Lanier et al., 1989a). CD3ζ is required for the development of T cells but apparently not of NK cells. NKT cells are defined as T cells bearing a unique invariant T cell receptor (TCR) plus NK1.1, whereas NK cells have only the latter surface marker (Matsumoto et al., 2000).

Subsets of NK cells can be distinguished by the surface density expression of CD56, as well as the presence and absence of CD16 (Caligiuri, 2008). Most of the peripheral blood NK cells are $CD56^{dim}$, which are rare in lymph nodes. $CD56^{dim}$ cells are functionally almost exclusively cytotoxic (Robertson and Ritz, 1990). $CD56^{bright}$ NK cells are predominant in secondary lymphoid tissues (lymph nodes, tonsils) and preferentially produce many cytokines. Notably, even the $CD56^{dim}$ is generally related to cells having natural killing capacity; the marker itself is not required for target recognition. CD56-positive NK cell subsets show differences also in their NK receptor repertoires (Lanier, 1998). $CD56^{bright}$ cells express high levels of C-type lectin-like CD94/NKG2 family with only few of the cell populations expressing killer-cell immunoglobulin-like receptors (KIR). Cytotoxic $CD56^{dim}$ NK cells express strongly both KIR and C-type lectin-like receptors (Farag et al., 2002). The phenotype of 'resting' NK cell resembles the effector CD8+ T cell: many NK receptors are expressed on CTL only after their maturation to effector or memory cells, whereas they are constitutively present on NK cells (Vivier and Anfossi, 2004).

2.3 NK cell activation

Lymphokine-activated killer (LAK) cells were first described by Rosenberg et al. in the early 1980s (Grimm et al., 1982; Grimm et al., 1983; Mule et al., 1984). When attempting to grow lymphoid cells that infiltrate solid tumors, they discovered that such cells, when incubated with the cytokine interleukin-2 (IL-2), acquired the ability to lyse a wide range of autologous and allogeneic tumors, although they spared normal non-malignant cells. In the '80s and '90s, a number of preclinical trials had shown that when LAK cells and IL-2 were infused into patients with metastatic cancer, impressive tumor regression could occur, and in some cases even tumor eradication. Clinical data have supported the contention that although NK and LAK activities seem to be similar with regard to their lytic properties, their mechanisms of target cell recognition and lytic attack appear to be quite different. LAK immunotherapy has been shown to have a potential to eradicate tumor cells in various forms of cancer (e.g. ovarian, malignant melanoma, breast and renal cell carcinoma) (Savas et al., 1996; Brinkmann et al., 1999; Savas et al., 2006).

NK cell response can be triggered within minutes, without requiring transcription, translation, or cell proliferation. As shown by Stetson et al., NK cells constitutively express prestored transcripts for interferon gamma (IFN-γ) that are immediately available to initiate cytokine synthesis upon activation (Stetson et al., 2003). NK activity can be increased up to 100-fold with cytokines, e.g. interferons alpha (IFN-α) and beta (IFN-β), and IL-12. These cytokines are produced early in many infections, and in addition to stimulating NK cytotoxicity they induce the production of other cytokines such as IFN-γ in NK cells themselves. $CD56^{bright}$ NK cells produce a series of cytokines, including IFN-γ, TNF-α, TNF-β, GM-CSF (granulocyte-macrophage colony stimulating factor), and IL-10 upon activation (Yokoyama et al., 2004). This suggests that NK cytokine production is also important in the initiation of acquired immune response. This makes NK cells well suited for early and immediate defense.

2.4 MHC class I specific receptors

For almost two decades NK cell killing has often been referred to as "non-MHC-restricted" cytotoxicity, in order to distinguish innate NK-mediated cytotoxicity from adaptive T cell function. This interpretation has recently been questioned, since NK cells express MHC class I-specific receptors, which have the ability to inhibit or activate NK cell function. There is no evidence that NK cells recognize MHC class II molecules. The data are based mostly on the analysis of class II-deficient mice, and on *in vitro* functional studies using target cells expressing MHC class II (Ohlen et al., 1995; Hayakawa and Smyth, 2006).

NK cell effector functions are regulated by a balance between activating and inhibitory signals through a wide repertoire of receptors (*Tables 1a and b*). Down-regulation of self-MHC class I molecules, up-regulation of ligands for activating receptors, or expression of pathogen-associated molecules (stress signals) can be recognized by NK cells leading to their activation or inhibition (Luci and Tomasello, 2008).

Table 1a. Activatory human NK cell receptors and their ligand specificities. Some candidate receptors (such as LFA-1) are also included. Other receptors such as growth factors and chemokines are not shown. Modified from (Lanier, 2005; O'Connor et al., 2007; Bryceson and Long, 2008; Cheent and Khakoo, 2009).

Activating Receptor	Ligand	Signaling
KIR2DS1	HLA-C2	DAP12-ITAM
KIR2DS2	HLA-C1	DAP12-ITAM
KIR2DS3	unknown	DAP12-ITAM
KIR2DS4	HLA-C	DAP12-ITAM
KIR2DS5	unknown	DAP12-ITAM
KIR3DS1	HLA-Bw4?	DAP12-ITAM
KIR2DL4	HLA-G (soluble)	ITIM, FcεRIγ
NKG2D	MICA, -B; ULBP	DAP10
NKG2E	HLA-E	?
NKp30 (CD337)	BAT3	TCRζ/FcRγ-ITAM
NKp44 (CD336)	cellular unknown, viral HA	DAP12-ITAM
NKp46 (CD335)	cellular unknown, viral HA	TCRζ/FcRγ-ITAM
CD16 (FcRγIII)	Fc of IgG	TCRζ/FcRγ-ITAM
DNAM-1 (CD226)	Necl-5 (CD155, PVR), nectin-2 (CD112)	?
NKp80	AICL	?
CD59	unknown	?
NTB-A	NTB-A	?
2B4 (CD244)	CD48	SAP
CD2	LFA-3 (CD58)	?
LFA-1 (CD11/CD18)	ICAM-1 , -2, -3, -4, -5	?
TLR	PAMPs, dsRNA	?
CD96 (Tactile)	Necl-5 (CD155)	?
CD44	HA, others?	?
CD94/NKG2C	HLA-E	DAP12-ITAM

KIR, killer cell immunoglobulin-like receptor (CD158); HLA, human leukocyte antigen; DAP12, ITAM-bearing transmembrane adaptor; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; NKG2, NK cell group 2 transmembrane receptor; MIC, MHC class I-chain-related molecule; ULBP, UL16-binding protein; BAT3, HLA-B-associated transcript 3; NKp, NK cell specific receptor; TCR, T cell receptor; HA, hemagglutinin; DNAM-1, DNAX accessory molecule-1; Necl-5, nectin-like molecule-5; AICL, activation-induced C-type lectin; PVR, poliovirus receptor; NTB-A, NK-T-B antigen (self-ligands); SAP, signaling lymphocytic activation molecule (SLAM)-associated protein; LFA, lymphocyte function-associated antigen; ICAM, intercellular adhesion molecule; TLR, toll-like receptors; PAMPs, pathogen-associated molecular patterns; dsRNA, double-stranded RNA; Tactile, T cell-activated increased late expression. • NK cells express three ITAM-containing adapter proteins: FcεRIγ, CD3ζ, and DAP10 (Anderson et al., 1989; Hibbs et al., 1989; Lanier et al., 1998). CD94/NKG2C and KIR molecules lacking ITIMs and having a charged residue in their transmembrane domains are likely to pair with the DAP12 signaling adapter molecule. KIR2DL4 is an exception: it has an ITIM in its cytoplasmic domain and is associated with the FcRIγ signaling adapter.

Table 1b. Inhibitory human NK cell receptors and ligand specificities. Modified from (Lanier, 2005; O'Connor et al., 2007; Bryceson and Long, 2008; Cheent and Khakoo, 2009).

Inhibitory Receptor	Ligand	Signaling
KIR2DL1	HLA-Cw2, -4, -5, -6	ITIM
KIR2DL3 (2DL2)	HLA-Cw1, -3, -7, -8	ITIM
KIR2DL5	unknown	ITIM
KIR3DL1	HLA-Bw4	ITIM
KIR3DL2	HLA-A3, A11	ITIM
KIR3DL3	unknown	ITIM
CD94/NKG2A	HLA-E	ITIM
ILT2 (CD85j, LILRB1)	HLA-A, B, C, E, F, G; CMV UL18	ITIM
ILT4 (CD85d, LIR2)	HLA	?
LAIR-1 ?	collagens?	ITIM
Sigleg-7	sialic acid	?
MAFA	cadherins	?
NKR-P1 (CD161)	LLT1	ITIM
CEACAM1 (CD66a)	CEACAM1	?

KIR, killer cell immunoglobulin-like receptor (CD158); HLA, human leukocyte antigen; ITIM, immunoreceptor tyrosine-based inhibitory motif; NKG2, NK cell group 2 transmembrane receptor; ILT, immunoglobulin-like transcript; LILR/LIR, leukocyte immunoglobulin-like receptor; CMV UL18, cytomegalovirus encoded glycoprotein; LAIR-1, leukocyte-associated immunoglobulin-like receptor-1; MAFA, mast cell function-associated antigen; NKR-P1, natural killer cell receptor protein 1; LLT1, lectin-like transcript 1; CEACAM, carcinoembryonic antigen-related cell adhesion molecule. • Inhibitory KIR, ILT2, and CD94/NKG2A contain ITIMs in their cytoplasmic domains.

The same molecule may sometimes serve both a triggering and an inhibitory ligand (*Tables 1a and b*). The reason for the same structure being activating as well as inhibitory may in part relate to the density of the molecules on the target cell, and to the activatory or costimulatory molecules that associate with the cytoskeletal proteins.

3. Cytotoxic mechanism

Cytolytic killing is a major effector mechanism in the elimination of tumor cells or virally infected cells. NK cells and CTL, despite their differential immune recognition, both have the same lytic mechanism of action, i.e. the cytolytic granule release.

NK cells can detect target-cell-bound antibodies by a mechanism called antibody-dependent cellular cytotoxicity (ADCC) (Perussia, 1998). The low-affinity receptor for IgG, CD16 expressed on NK cells, mediates ADCC and signals through adaptors containing a cytoplasmic immunoreceptor tyrosine-based activatory motif (ITAM), which activates the cytotoxic machinery.

The mAbs used for anti-cancer therapies can effectively stimulate NK cell activity through CD16. Freshly isolated NK cells are poorly cytotoxic, but their cytotoxicity is strongly enhanced by using mAb-coated target cells. It seems that therapeutic mAbs against tumors rely on the ability of NK cells to sequentially kill numerous target cells (serial killing). It is noteworthy that most of the effectiveness of widely used successful therapeutic antibodies, such as rituximab (anti-CD20) and trastuzumab (herceptin, anti-erB2) – to treat patients with lymphoma or breast cancer, respectively – is based mainly on the cytotoxic function of NK cells (Adams and Weiner, 2005; Carter, 2006). Recent *in vitro* data indicate that some cancer cells, especially cancer stem cells, may evade ADCC due to the lack of an antibody ligand. The tumor-initiating cells are thus spared, and this could explain the clinical relapse and progress of the disease (Reim et al., 2009).

NK cells nevertheless lyse targets and function well even in the absence of antibodies. Natural cytotoxicity involves a coordinated series of events, starting from contact with target cells, tight adhesion, synapse formation, granule polarization and granule exocytosis (Orange, 2008). Resting NK cells make use of preformed lytic granules to kill target cells. This cytolytic effect is rapid.

Granule release – perforin and granzymes

Killing is mediated by cytotoxic molecules which are stored within secretory vesicles, specialized exocytic organelles found in NK cells. The cytotoxicity of NK cells is executed through the granule exocytosis pathway by which the perforin and granzyme B content of granules is released into the immunological synapse (IS) after conjugate formation with the target (Lieberman, 2003). Granzymes are secreted into a small enclosed space (in IS), and only minute amounts of cytolytic material leak out into the extracellular space. During chronic inflammation (e.g. rheumatoid arthritis joints) granzyme A can reach low-

nanomolar levels in extracellular fluids. Normal concentrations in the blood are below 1pM (Spaeny-Dekking et al., 1998). The granule content released into a single synapse has been estimated to be vastly greater, up to 8μM. The local synapse granzyme A concentration is sufficient for lysis to occur, being activated at 250nM (Fan et al., 2003; Martinvalet et al., 2008).

The cytotoxicity proceeds in a series of steps: the NK cell first binds the target cell, forms a conjugate, delivers a lethal hit and disassociates from the target. After the dissociation of conjugates, the NK cell can repeat the same cycle. A single NK cell may kill four, or even up to six target cells in 'in a row' (Bhat and Watzl, 2007). Interestingly, when NK cells and CTL were studied in target cell contacts, the majority of the NK cells showed stepwise and slow cytoskeletal polarization, whereas the CTL established cytoskeletal polarization rapidly (Wulfing et al., 2003). Furthermore, NK cell killing was particularly sensitive to minor disturbances in cytoskeletal dynamics. The authors suggested that the stepwise cytoskeletal polarization serves several important checkpoints in NK cell killing.

The cytotoxicity of NK cells is mostly mediated by the release of perforin and granzyme B. It has been shown that NK cells undergo functional exhaustion after killing, leading to a partial loss of perforin and granzyme B. This may be restored by IL-2. Furthermore, it has been observed that a single NK cell was able to make contact with two or more targets at the same time, but the targets were never killed simultaneously. Only very few dead NK cells were detected in conjugates, indicating that a great majority of the NK cells was resistant to cytotoxicity. Another study showed that about 50% of conjugates between IL-2-activated mouse NK cells and NK-sensitive targets were dissociated without killing, implicating that conjugate formation without ensuing lysis can occur (Wulfing et al., 2003).

4. Target cell recognition

Target cell recognition induces the formation of a lytic immunological synapse between the NK cell and its target. To ensure that NK cells do not kill

indiscriminately and are able to distinguish between healthy and diseased cells, the entire cytotoxic process is a strictly regulated and highly ordered process.

Target cell recognition can be divided into different processes: 1) binding of effector cells to the target, 2) rearrangement of the actin cytoskeleton, 3) forming of an activating, lytic immunological synapse, 4) polarization of the microtubule-organizing centre (MTOC) of the NK cell and the secretory lysosomes towards the lytic synapse, 5) docking the secretory lysosomes with the plasma membrane at the lytic synapse, 6) fusion of the secretory lysosomes and release of their cytotoxic contents, and 7) dissociation of the effector cell from the target (Davis, 2009). The concept target cell recognition can be defined as regulation of the effector cell cytolytic machinery mediated by target-cell-recognizing NK cell receptors (Helander and Timonen, 1998).

4.1 Binding to a target – first requirement, but is it sufficient for triggering?

The adhesion of lymphocytes to other cells and tissues is essential for recognizing and killing of target cells, and also for hematopoiesis, communication with APC, immune surveillance, and multiple steps of lymphocyte recirculation (Springer, 1990; Dustin et al., 1992).

LFA-1 on the surface of effector cells

Target cell binding is the first necessary, but probably not sufficient, step in NK cell cytolytic activity. In this process, the integrins and immunoglobulin superfamily molecules are crucial (Orange et al., 2003). Leukocyte integrin LFA-1 is a heterodimeric receptor composed of an integrin alphaL chain (CD11a) that associates with an integrin β2 chain (CD18) (Wang and Springer, 1998). LFA-1 is expressed on all lymphocytes, granulocytes, monocytes, macrophages and NK cells. LFA-1 interacts with ICAMs (ICAM-1 to -5), ligands that are differentially expressed on leukocytes, endothelial cells, epithelial cells and fibroblasts (Staunton et al., 1989). LFA-1 also regulates leukocyte adhesion to the endothelium and extravasation and has a role in T cell co-stimulation during activation.

CD18 is the major adhesion structure of fresh, endogenous (unstimulated) NK cells. MAbs against CD18 can inhibit T cell proliferation, adhesion of CTL and NK cell cytotoxicity (Chen et al., 1987; Timonen et al., 1988). In the absence of LFA-1 engagement, seen in patients with CD18-deficient leukocyte adhesion deficiency (LAD), target cell lysis by cytotoxic lymphocytes is impaired (Kohl et al., 1984). This is also seen in mice with targeted mutations in CD11a or CD18, or after blocking of LFA-1 with mAbs (Schmits et al., 1996). NK cells from LFA-1-deficient mice are unable to kill target cells due to impaired binding capacity (Matsumoto et al., 1998).

Matsumoto et al. showed that LFA-1 regulates the cytotoxicity of IL-12-activated NK cells against tumor targets, and that the adhesion was impaired in the absence of LFA-1. They also studied conjugate formation, and found that LFA-1-mediated adhesion seemed to be crucial for NK-mediated cytotoxicity, and that it is required to sustain conjugates long enough for interactions between specific surface receptors to initiate cytolysis. LFA-1 expression was generally required for optimal conjugate formation between cytokine-activated NK cells and target cells. LFA-1 thus has an important role in the cytotoxicity of LAK cells (Matsumoto et al., 2000). LFA-1 also induces perforin polarization in NK cells.

Barber and colleagues showed that LFA-1 alone was sufficient to initiate activation signals in NK cells, in contrast to T cells in which signals from other receptors were needed even though adhesion was mediated through LFA-1 (Barber et al., 2004). It seems that adhesion molecules not only mediate the binding but may also act as activating molecules in NK cell killing.

ICAM-2 on target cells

Currently five intercellular adhesion molecules, ICAM-1–5, are known. They mediate cell adhesion by binding to LFA-1. The expression of ICAM-1 is sufficient to induce lysis by IL-2-activated NK cells through LFA-1. ICAM-1 (CD54) is the most widely distributed ICAM; it is found on the surface of leukocytes, endothelial cells and other cells (Nortamo et al., 1991). It has been shown that the expression of ICAM-1 is up-regulated by several proinflammatory cytokines (including IFN-γ), and by infection of endothelial and epithelial cells

with bacteria and viruses (Shen et al., 1997; Frick et al., 2000). ICAM-1 may also act as a signaling molecule, and the expression of ICAM-1 alone is sufficient to initiate signal-dependent adhesion on resting NK cells. This adhesion was found to be strongly enhanced by either a pulse of IL-2 or IL-15, or the coexpression of LFA-3 or CD48 with ICAM-1 on target cells (Holland and Owens, 1997; Barber and Long, 2003).

The integrins can be activated by variety of agents, and at least two major pathways of activation are known. Activation can be as a result of increasing the affinity of the molecule, probably via conformational changes, or by increasing the avidity of adhesion, which may result from an increase in the number or clustering of integrins in the cell membrane.

ICAM-2 (CD102), like ICAM-1, is relatively broadly distributed in different tissues, but low levels are constitutively expressed on most leukocytes, endothelial cells and platelets (Nortamo et al., 1991; Diacovo et al., 1994). It seems to be involved in lymphocyte recirculation, trafficking, and extravasation (Gerwin et al., 1999). Other ICAMs have a more restricted expression. ICAM-3 (CD50) is present in large quantities on resting lymphocytes, monocytes and granulocytes, and is the only ICAM significantly expressed on neutrophils (de Fougerolles and Springer, 1992). The expression of ICAM-4 (CD242) is restricted to red blood cells, and ICAM-5 (telencephalin) is expressed in the brain (Bailly et al., 1994; Yoshihara et al., 1994). All ICAMs may at least partially substitute for each other because no disease has been described in which where ICAMs would be absent or defective.

CD2–LFA-3 pathway

Several adhesion molecules participate in the binding of the effector cell to the target (Timonen and Helander, 1997). CD2 also activates NK cell cytotoxicity and cytokine production, but the function in NK activity may be merely co-stimulation. Its ligand LFA-3 (CD58), is expressed on the cell surface in both a transmembrane and a glycosylphosphatidylinositol (GPI)-anchored form (Dustin et al., 1987; Davis et al., 1998). In addition to cell adhesion, CD2 is generally

considered to be a costimulatory molecule, and involved in signaling (Springer et al., 1987).

Kaizuka et al. recently showed that a native GPI-anchored form of LFA-3 could trigger a downstream signaling cascade through the CD2 receptor in the absence of TCR (Kaizuka et al., 2009). Interestingly, they further showed that this signaling process was accompanied by a dramatic actin-dependent reorganization of CD2 and downstream signaling molecules into micro-domains. They found that CD2 and TCR were stimulated together, and co-clustered at the cell periphery, but segregated from one another near the cell center. They suggested that this local cluster of CD2–LFA-3 and TCR together increases the concentration of signaling molecules, which can then trigger T cell signaling.

4.2 Triggering of lysis

It is still uncertain whether adhesion molecules can also function as direct triggering receptors. NK cell receptors recognize their ligands on target cells and lead to a series of intracellular transduction (*Table 1*). The different NK receptor-ligands can activate distinct signaling pathways, including extracellular signal-regulated kinase (Erk1/2) and c-Jun NH2-terminal kinase (JNK). If the NK-target-cell conjugate is impaired, the signaling pathway is not activated, and NK cell cytotoxicity is inhibited (*Table 2*). It has been shown, however, that if the signal pathways are inhibited and NK cell cytotoxicity is suppressed, the NK-target conjugate formation may still remain intact (Zheng et al., 2009).

Because of the multiplicity of receptor-ligand interactions between NK cells and target cells, it has been difficult to specify distinct functions to individual activating receptors, or to assess the outcome of the simultaneous engagement of defined combinations of receptors. It is still not clear which receptors mediate signals for adhesion and for granulate exocytosis during natural cytotoxicity, or which signaling events are induced by inhibitory receptors, and whether the activation receptors differ regarding their sensitivity to inhibition. A recent study focusing on the minimal receptor-ligand interactions required to cause natural cytotoxicity revealed that strong inside-out signals are induced by a combination

of NKG2D and 2B4 or by CD16, which can overcome CD94/NKG2A inhibition (Bryceson et al., 2009).

4.3 Immunological synapse – the time and place of decision

Almost 30 years ago, long before the immunological synapse (IS) was defined as a biological unit, the polarization of the NK cell cytoskeleton and the cell-cell contacts were described (Carpen et al., 1982; Carpen et al., 1983). The authors analyzed various characteristics of the contact area of human NK cells with a target by using scanning and transmission electron microscopy (SEM and TEM). They studied cytoskeletal changes in NK cells during conjugate formation, and emphasized the importance of a specific cell contact site where cytoskeletal proteins were polarized (reorganization of actin and vinculin). Also the polarization of the Golgi apparatus towards the contact area was seen after cell-cell contact.

Table 2. Defects of the lytic synapse in NK cells from patients with a genetic disease. Modified from Orange (2008).

Disease	Protein	Effect on immunological synapse (IS)
LAD-I	CD18	decreased conjugation with target
WAS	WASP	decreased F-actin reorganization and integrin distribution
CHS	LYST	inability to generate normal lytic granules for trafficking to the IS
HPS2	AP3 β subunit	inappropriate formation of lytic granules and movement along microtubules
GS2	RAB27A	lytic granules move to the synapse but remain associated with microtubules
FHL3	MUNC13-4	lytic granules move to the IS, but fail to dock and so do not achieve an intimate association with the NK-cell plasma membrane
FHL4	Syntaxin-11	lytic granules polarize to and dock at the NK-cell plasma membrane but fail to fuse

LAD-I, leukocyte adhesion deficiency type I; CD18, β_2-integrin; WAS, Wiskott-Aldrich syndrome; WASP, WAS protein; CHS, Chediak-Higashi syndrome; LYST, lysosomal trafficking regulator; HPS2, Hermansky-Pudlak syndrome; AP3, adaptor protein 3; GS2, Griscelli syndrome type 2; FHL, familial hemophagocytic lymphohistiocytosis.

Actin cytoskeleton

Actin is a major cytoskeletal protein. It is extremely well conserved in eukaryotes, with little variation in sequence, but due to the great variety of actin-associated proteins it plays many roles, from cell shape maintenance to cellular motility (Gerwin et al., 1999). In most cells actin is highly concentrated in the immediate vicinity of the membrane: the assembly and disassembly of actin are controlled by events at the membrane. Controlled polymerization or depolymerization of actin filaments is required not only for the production of filaments needed for interaction with other components of the cytoplasm, but also for many other kinds of cellular activity (e.g. protrusion of membranes, activation, effector functions, deadhesion, cell locomotion and migration) (Burkhardt et al., 2008).

Ezrin, a linker protein between plasma membrane and actin cytoskeleton

The ezrin-radixin-moesin (ERM) protein family is a group of highly related molecules that participate as linker structures in membrane-cytoskeleton interactions (Arpin et al., 1994). Ezrin (first named cytovillin) was independently discovered and sequenced at the Haartman Institute, University of Helsinki, and at the Cornell University (Bretscher, 1983; Suni et al., 1984). Ezrin is localized in microvilli and other cell surface structures (filopodia, membrane ruffles). The ezrin gene was located to long arm of chromosome 6 (6q22-q27) (Turunen et al., 1989; Majander-Nordenswan et al., 1998). It has been shown that in virus infections ezrin is redistributed from diffuse localization to newly formed microvilli (Pakkanen et al., 1988).

Proteins homologous to ezrin have been discovered: moesin and radixin are associated also with cell surface structures and they co-localize with actin (Tsukita and Hieda, 1989; Sato et al., 1992). The actin-binding site of ezrin was mapped to the COOH-terminal 34 aminoacids of ezrin, and later the same region was confirmed as a binding site for moesin and radixin (Turunen et al., 1994; Henry et al., 1995; Pestonjamasp et al., 1995). In addition, ezrin has been shown to bind cell adhesion molecules CD44, ICAM-1, ICAM-2 and seems to regulate cellular adhesion, which is crucial in metastasis (Tsukita et al., 1994; Heiska et al., 1998).

Ezrin is expressed in several human tumors, including intestinal tumors, salivary gland tumors, renal cell adenocarcinoma and hemangioblastoma (Bohling et al., 1996).

5. Virus infections and NK sensitivity

Virus-infected cells produce IFN-α and -β which induce NK cell proliferation and cytotoxicity. NK cells secrete large amounts of IFN-γ in response to IL-12 and IL-18 which are produced during infections by infected cells as well as cells of the immune system. Some viruses alter the expression of MHC class I molecules quantitatively by directly down-regulating cell surface expression and/or by interfering with the ability of IFNs to up-regulate class I molecules (Brutkiewicz and Welsh, 1995).

One way to evade the NK-cell-mediated response is by mimicking or blocking the MHC molecule (such as CMV, which blocks HLA-E expression) (Kavanagh et al., 2001; Lanier, 2008). Human CMV encodes glycoprotein (UL18) which is structurally similar to MHC class I, and it affects the NK response by binding to the NK inhibitory receptor. These homologs of the MHC class I may serve as decoy ligands, allowing the virus to hide from NK cells. However, the activating receptor on the NK cell may bind to CMV peptide complex and override the effect of the inhibitory receptor, thus leading to NK cell activation.

It has been suggested that the influenza virus hemagglutinin could serve as a triggering ligand for NK cells (Mandelboim et al., 2001). The authors showed that an activating receptor on NK cells, NKp46, bound to the hemagglutinin of the influenza virus, and consequently blocked the killing partially. The other activating receptor, NKp30, has been shown to associate with the tegument protein pp65 of CMV, but this interaction suppressed the NK cell cytotoxicity (Arnon et al., 2005).

The rarity of viral structures in the activation of NK killing was somewhat unexpected, since the major biological role of NK cells has been shown to be resistant against viral infections (Cerwenka and Lanier, 2001).

Aims of the Study

The general aim of this study was to investigate target cell sensitivity to NK activity.

More specifically

to identify regulatory elements and gene(s) that sensitize target cells to IL-2-activated killer cells, and

to provide a physiological model, i.e. viral infection, to clarify molecular mechanisms involved in the sensitization process.

Summary of the Materials and Methods

An overview of the materials and methods of this study is presented in the following sections, and are all listed in Table 3. More detailed technical descriptions (e.g. reagents, antibodies used) can be found in the original publications which are referred to by Roman numerals (I-III). One method not described in the original articles is presented (unpublished results), and is referred to in the original papers listed in the References.

Effector cells (I-III). Several different effector cells and cell lines were used. Fresh effector cells were isolated and purified using several techniques. Lymphocytes from healthy blood donors were obtained as buffy coats from the Finnish Red Cross Blood Service. The cells were isolated by Ficoll-Isopaque density gradient centrifugation and filtration through nylon wool columns (PBL). NK cells were mainly enriched by Percoll gradient centrifugation (Timonen and Saksela, 1980). To isolate different lymphocyte subsets (NK, Th, Ts, T cells) the isolated PBL were labeled with mAbs and separated using magnetic beads (immunomagnetic cell sorting). CD3-positive T cells were isolated by negative or positive selection using CD56 or CD3 coated beads, respectively. When positive selection was used, the cells were detached by incubation overnight at +37 °C. The other cell subsets were isolated by negative selection; the cells that were attached to the beads were removed and the remaining cells were used in the assays. The efficiency of the cell isolation was confirmed by flow cytometry (FACScan). PBL were incubated with 500 IU/ml rIL-2 overnight to generate the LAK activity (LAK cells). Also mouse killer cells isolated from AKR mouse spleen were used as effectors in the cytotoxicity assays (both non-activated and IL-2-activated cells were used).

Target cells (I-III). (I-II) Somatic cell hybrids between mouse (BW5147 or NS-1 cells) and human lymphocytes were used as target cells. K562 cells served as a positive control (NK-sensitive cell line). (III) Infected or transfected HeLa cells were used to study the effect of various viral ns-proteins on NK-sensitivity. The HeLa cells were grown on cover slips, or on flat-bottom 96-well microtiter plates for the cytotoxicity assays. The mock-infected and vaccinia-virus-infected HeLa cells served as controls.

Chromosome analysis of mouse/hybrid cells (I-II). For the karyotypic analysis, chromosome preparations were stained with a modification of the trypsin-Giemsa banding method (Seabright, 1971). Because the karyotypes of the clones are not stable and they tend to lose human chromosomes in culture, the chromosome preparations were made simultaneously with the functional experiments, i.e. binding and cytotoxicity assays. At least 30 cells (metaphases) were analyzed from each hybrid cell clone. The hybrids used contained human chromosomes in 65–90% of the cells. Also the viability of the cells was assessed by the trypan blue test.

Chromosomal *in situ* suppression hybridization, CISS (unpublished). Hybrid clones and the parental cells were painted with the human chromosome 6 specific probe, and also with the whole human genomic DNA. CISS hybridization was performed with biotin-labeled total human genomic DNA and bacteriophage DNA library of human chromosome 6 (ATCC LL06NS01) as described by Lichter et al. (1988). Prior to the hybridization, the cytoplasm was removed by treating the slides with pepsin digestion. The probes were detected by FITC-conjugated avidin, and the signals were amplified (Pinkel et al., 1986). Cells were counterstained with propidium iodide and DAPI (4´,6-diamidino-2-phenylindole-dihydrochloride) and mounted in fluorescence antifading buffer (Johnson and Nogueira Araujo, 1981). The chromosome painting was performed in collaboration with Dr. Marianne Tiainen, University of Helsinki.

Binding assay (I). Effector cells were mixed with target cells and centrifuged, and the cell pellet was then resuspended by three gentle aspirations through a Pasteur pipette. The cell suspension was pipetted on an objective slide, and the percentage of binding lymphocytes and the total number of lymphocytes were immediately

calculated by light microscopy. 100 effector cells, regardless of binding, were counted and the results were based on 3–8 different experiments.

Cytotoxicity assay (I-III). Target cells were labeled with sodium ^{51}chromium (^{51}Cr), washed and incubated with effector cells at three different effector:target cell ratios in 96-well microtiter plates. The supernatant from each well was collected and the radioactivity was measured in a gamma-counter. For inhibition assays the cells were pretreated with mAbs.

Flow cytometry (I-II). To study the level of expression of the surface molecules, the cells were labeled with various antibodies, and subsequently incubated with FITC-conjugated secondary antibody. Isotype-matched Igs were used as negative controls. The fluorescence was determined by FACScan.

Immunofluorescence microscopy (I-III). To study the cell surface expression and distribution of an antigen, the cells were incubated with normal rabbit serum, washed, incubated with the primary antibody (Ab), and after washing incubated with FITC- or TRITC-conjugated secondary Ab. For staining the proteins in cytosol, the cells were fixed, permeabilized and treated with primary antibody (e.g. human or mouse ezrin, or viral nsP-antibodies; and for visualization of F-actin the phalloidin was used), followed by FITC- or TRITC-conjugated secondary Ab. The nonspecific staining was controlled using the isotype-matched immunoglobulins. Also the binding of the secondary reagents in the absence of the primary antibody was tested.

Phase-contrast microscopy (II). To study the binding and to visualize the effector-target cell conjugates, the LAK cells were mixed with the target cells and incubated for 1h. They were then cytocentrifuged gently, analyzed and photographed under a phase-contrast microscope.

Immunoblotting (II). For Immunoblotting, proteins separated by SDS-PAGE were transferred to nitrocellulose membranes. To study the expression of human and mouse ezrin, the human-specific ezrin antibody (CVP1) and the ezrin antibody recognizing both mouse and human ezrin (3C12) were used. Human JEG-3 cells served as a positive control.

Transfection experiments (II-III). (II) Human ezrin cDNA was subcloned into the pcDNA3 expression vector and the cells (BW5147, 293) were transfected by using DOTAP transfection reagent. To achieve efficient and rapid expression of various nsPs, the HeLa cells were first infected with the recombinant vaccinia virus system vTF7-3 and then transfected with plasmids containing the desired nsP-encoding gene under T7 promoter in pGEM3 using Lipofectin (Fuerst et al., 1986). Hydroxyurea was used simultaneously with Lipofectin to inhibit vaccinia virus DNA replication. Transfection efficiency varied between 80−100%. (III) For transient expression of nsP1 under CMV promoter, HeLa cells were transfected with nsP1-PC1 plasmid using Lipofectin according to the manufacturer's instructions.

SFV infection (III). HeLa cells were infected with wt SFV using 200 PFU/cell as described previously (Laakkonen et al., 1998).

SEM (III). HeLa cells grown on cover slips and then transfected were stabilized in the presence of unlabeled phalloidin in a cytoskeleton-stabilizing buffer according to Singer et al., and as modified by Sormunen (Singer et al., 1989; Sormunen, 1993). The cells were fixed and dehydrated in ethanol and hexamethyldisilatzane. Cover slips were coated thinly with platinum and examined under a digital scanning microscope. In order to count the number of NK cells bound to one target cell, IL-2-activated PBL were added to the target cells and incubated for 2 h before fixation.

Statistical analysis (I-III). Mean values and statistical differences were calculated from the results of 3 to 21 experiments (i.e. different lymphocyte donors). Values were calculated with parametric two-tailed Student's test in order to show differences in the binding or cytotoxicity of effector cells from the same donor. The non-parametric Mann-Whitney U test was used to compare different donors. In addition, several experiments were conducted because of the variation (individual differences) in the killer cell (basic) activity of the lymphocyte donors.

Table 3. The materials and methods used in this study.

Material or assay used	Described in paper
Materials	
Effector cells	
PBL, LAK	I-III
NK	I-III
T, Ts, Th	I
mouse killer cells (from AKR mouse spleen)	II
Target cells	
human/mouse hybrid clones	I-II
BW5147 (AKR mouse thymoma)	I-II
NS-1 (BALB/c myeloma)	I
K562 (chronic myelogenous leukemia)	I-II
293 (human embryonic kidney-derived cells)	II
JEG-3 (human choriocarcinoma)	II
HeLa (cervix carcinoma)	III
Methods	
Isolation of lymphocytes	I-III
Cell cultures	I-III
Chromosome analysis	I-II
Chromosomal in situ suppression hybridization	unpublished
Binding assay	I-II
^{51}Cr-release cytotoxicity assay	I-III
Competition experiment	I
Blocking of effector cell functions	I-III
Cell transfection	II-III
Cell infection (VV, SFV)	III
Immunofluorescence microscopy	II-III
Flow cytometry	I-II
Immunoblotting	II
Scanning electron microscopy (SEM)	III
Statistical analysis	I-III

Results and Discussion

The Roman numerals refer to the original publications.

1. IL-2-activated NK cells and human chromosome 6 (I)

1.1 Mouse/human cell hybrids as target cells

At the time when this study was initiated very little was known about the target structures recognized by NK cells. We studied the differences and capacity of unstimulated and IL-2-activated NK cells to bind and kill target cells, and used mouse/human hybrid cells containing various human chromosomes as targets. Hybrid cells have earlier been used successfully as a tool for assigning human genes (gene mapping) to human chromosomes. We analyzed several hybrid clones ('karyotyped' human chromosomes) containing from one single human chromosome to eleven different human chromosomes. The human genomic material on the hybrids and parental cells was further verified by chromosomal *in situ* suppression hybridization (CISS) method. The 6-hybrid cells contained one or two human chromosomes number 6 per cell (in at least 80% of cells). Parental cells were negative for both chromosome 6-specific and total human DNA probes (*Fig. 2*).

1.2. Binding of non-activated and IL-2-stimulated PBL to mouse/human hybrid cells

The percentage of effector cells that bound to target cells was analyzed. The binding of unstimulated PBL to hybrid cells was only slightly more effective than the binding to the mouse parental cells. However, about 30% to over 50% of the

LAK cells were bound to human chromosome 6 containing hybrid cells, compared to parental cells or hybrid clones having other than number 6 human chromosomes. Furthermore, the number of NK cells binding to the 6-hybrids was the same (or even higher) as that binding to K562 cells, which are known as NK-sensitive target cells and are usually used as a positive control in many (especially cytotoxicity) assays.

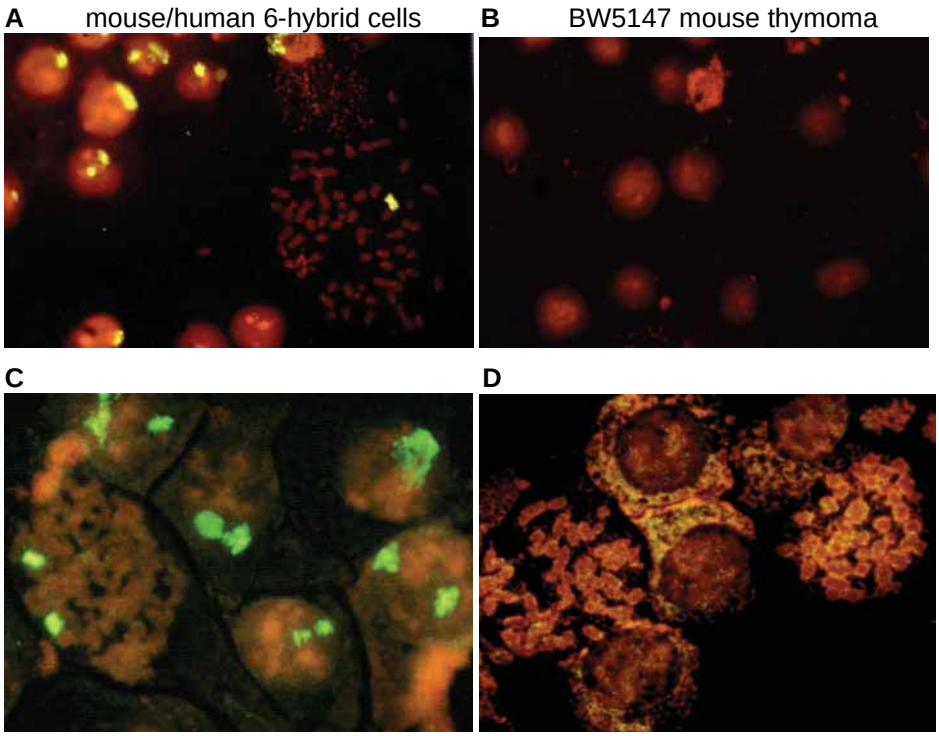


Figure 2. 6-hybrid clones (A, C) and parental cells (BW5147; B, D) painted (yellow) with biotinylated chromosome 6-specific library probes (A, B) and with total human genomic DNA (C, D).

To discover which subpopulation of lymphocytes binds to human chromosome 6 hybrid cells, the various lymphocyte subsets were used as effectors against the hybrid clones. Chromosome 6 was the only human chromosomal material in the 6-hybrid clones used as targets. Almost the same number of unstimulated NK cells, as of unstimulated PBL, was bound to target 6-hybrid cells. However, when activated by IL-2, 56% of the NK cells were bound to human chromosome 6 hybrids, whereas significantly fewer T cells were bound (only half of the number of NK cells; the subsets of T cells were bound at the same level as when they were

non-activated). It seems that mainly NK cells were responsible for LAK cell binding to chromosome 6-hybrid cells.

1.3 Cytotoxicity of non-activated and IL-2-stimulated PBL to 6-hybrid cells

In line with the results on binding, the unstimulated PBL killed hybrids or parental cells only weakly. In contrast, the hybrid cells were very effectively lysed by IL-2-activated killer cells. Even at the effector:target ratio 15:1 almost half of the hybrid cells were killed in a 3-h chromium release assay. Unstimulated T cells bound to the 6-hybrids, but were not able to lyse them at the same level as NK cells even after being activated by IL-2. NK cells effectively bound to and lysed the 6-hybrid cells. In other words, human chromosome 6 must encode genes important for IL-2-activated NK cell binding and cytotoxicity.

Because both the 6-hybrids and K562 cells were effectively bound to and killed by IL-2-activated NK cells, we wanted to find out whether different recognition mechanisms (structures) were involved in the killing of these two targets. We conducted a competition assay in which K562 cells compete with the 6-hybrid cells. We observed that K562 cells inhibited the 6-hybrid killing suggesting that K562 and 6-hybrid cells possibly share the same structures recognized by IL-2-activated NK cells.

1.4 Expression of human MHC class I and II antigens

It has been shown that the expression of human MHC class I antigens is central in NK response. The down-regulation of HLA antigens, e.g. as a result of viral infection, activate NK cell killing, whereas the increased expression of MHC class I inhibits NK activity. The human MHC cluster is located on the short arm of chromosome 6 (6p21). All hybrid cells carrying human chromosome 6 expressed both MHC class I and class II molecules. Our data thus imply that there may be other molecules that override the effect of the MHC inhibition.

Our group has demonstrated that the receptors involved in binding IL-2-activated NK cells to colon carcinoma cells were LFA-1 (CD11a-c/CD18) and CD2, CD54

and RGD-peptide-containing structures (Timonen et al., 1990). However, these could not be the target structures encoded on chromosome 6 since their genes are localized elsewhere. Also the other CD11/CD18 ligands were considered to be unimportant because these adhesion ligands seem to play only a minor role in IL-2-activated killing.

Also the gene for TNF-α is assigned to human chromosome 6 and is known to be important in NK cell killing. We cultured the parental cells with exogenous TNF-α to study whether this would increase binding and/or sensitivity. Parental cells were not sensitized to LAK cells in the presence of TNF-α.

Interestingly, it has earlier been shown that the introduction of a single human chromosome 6 into a human melanoma cell line (by microcell fusion) reduces the tumorigenicity of these cells in nude mice (Trent et al., 1990). The authors suggested that the genes in chromosome 6 suppress the malignant phenotype. One may speculate that the more benign phenotype may at least partially be due to the increased NK sensitivity of melanoma cells.

We conclude that human chromosome 6 sensitizes NK-resistant mouse cells to NK activity, and suggest that chromosome 6 contains gene(s) encoding either target ligands or regulatory molecules that control the expression of target ligands for IL-2-activated killer cells.

2. ICAM-2 and cytoskeletal ezrin in NK cell activity (II)

2.1 Adhesion molecules

The central receptor-ligand pairs in cell adhesion between target and effector cells are LFA-1–ICAMs and CD2–LFA-3. The effector cells were pretreated with mAbs against LFA-1 α-chains, CD11a, b, and c, and CD18 (β-chain) to inhibit the killing. The cytotoxicity was effectively blocked by CD11a and CD18 mAbs (present on killer cells). Also the mAbs against mouse ICAM-2 (the LFA-1 ligand present on target cells) inhibited killing. Other mAbs against the α-chains (CD11b–c) did not block the cytotoxic effect, showing that the CD11a/CD18-ICAM-2 pathway seems to be crucial in the recognition of chromosome 6 cell

hybrids by IL-2-activated killer cells. Also the activated murine killer cells that were isolated from AKR mouse spleen lysed the 6-hybrid cells, whereas the parental cells were resistant.

2.2 Expression and distribution of mouse ICAM-2, and cell polarization

Regulating the level of expression is one way to impact the function of the molecule. We studied the expression of mouse ICAM-1 and -2 by flow cytometry. The 6-hybrid and parent cells expressed equal levels of mouse ICAM-2. However, the distribution of mouse ICAM-2 on the cell surface was different: ICAM-2 was evenly distributed in NK-resistant mouse cells, but was strongly polarized in NK-sensitive 6-hybrid cells. Furthermore, 6-hybrids differed morphologically, because they had a prominent uropod (bud-like extension) on the tip of which almost all ICAM-2 expression was concentrated. The parental cells lacked these extensions. ICAM-1, another ligand of the CD11a/CD18 molecule, was evenly distributed in both cell lines.

The results show that ICAM-2 was redistributed in mouse BW5147 cells as a result of the introduction of human chromosome 6. Phase contrast microscopy revealed that 70% of the conjugates were uropod-associated. This indicates that the majority of IL-2-activated killer cells recognize structures expressed on the uropods. The ICAM-2 appears to have a key role in recognition, because ICAM-2 antibodies inhibited the cytotoxicity of the 6-hybrids. The redistributed ICAM-2 seemed to be involved in the recognition of the 6-hybrids by IL-2-activated killer cells.

2.3 Colocalization of membrane-cytoskeletal linker protein ezrin and ICAM-2

It is evident that chromosome 6 must carry genes that are important in sensitizing hybrid cells to IL-2-activated killing, and that ICAM-2 is a target structure in this process. However, the gene of ICAM-2 is assigned to human chromosome 17 (17q23-q25). So it is likely that there could be some regulating elements, encoded by genes located in chromosome 6 that are essential in the redistribution of ICAM-2.

It is known that the cytoskeleton has a central role in the topography of surface molecules. ERM family members act as molecular linkers between the cell surface protein CD44 and actin-based cytoskeleton (Arpin et al., 1994; Tsukita et al., 1994). The gene of one member of the ERM family, ezrin, has been localized on the long arm of human chromosome 6 (6q22-q27), and it has been proposed to act as a membrane-actin cytoskeleton linker (Turunen et al., 1989; Algrain et al., 1993; Majander-Nordenswan et al., 1998). Immunoblotting showed that both the 6-hybrid and parental cells expressed mouse ezrin. Furthermore, 6-hybrid cells expressed also human ezrin, which was verified using specific human ezrin antibodies. Analyzing the expression of ezrin by immunofluorescence microscopy revealed that the expression was similar to that of ICAM-2: also ezrin was localized to uropods in 6-hybrids, and was evenly distributed in parental cells (distribution of F-actin and ezrin, *Fig. 3*). When cytochalasin B was used to disrupt the F-actin cytoskeleton, the uropods and concentrated expression of ICAM-2 disappeared (data not shown).

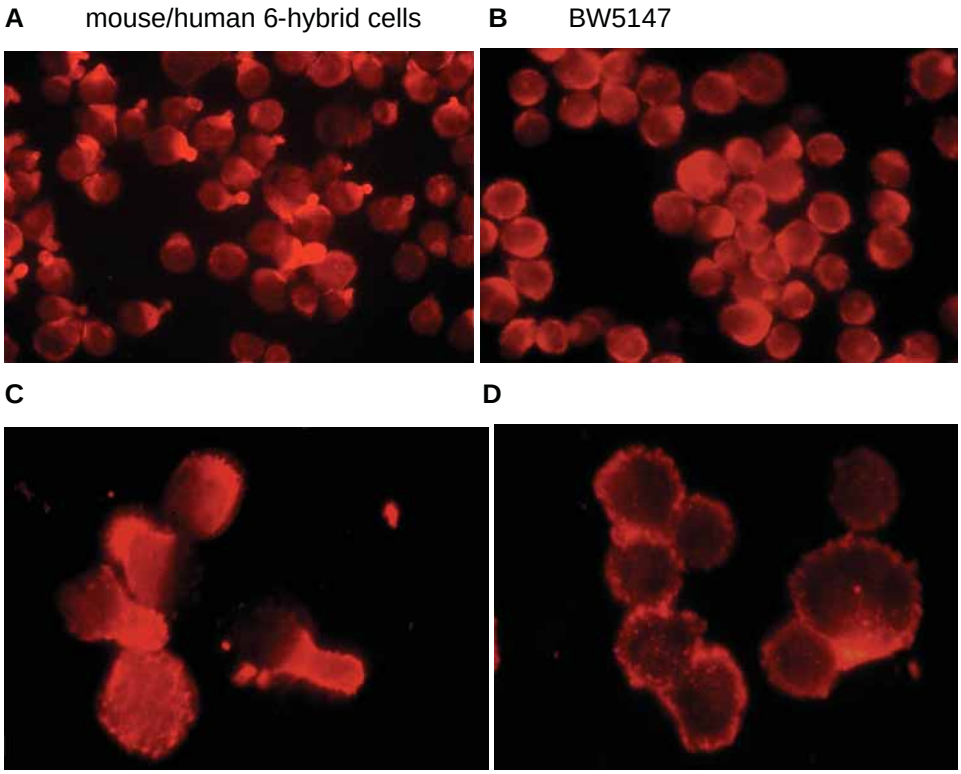


Figure 3. Localization of F-actin (phalloidin) (A-B) and ezrin (C-D) in 6-hybrids (A, C) and mouse parental, BW5147 cells (B, D). Note the prominent uropods in 6-hybrid cells where almost all actin and ezrin is concentrated.

These results suggest that the distribution of ICAM-2 is controlled (regulated) by the cytoskeleton, and ezrin plays a key role in redistribution cell-surface molecules into the newly formed cellular protrusions. Presently, a uropod is defined as a plasma membrane protrusion in which specific organelles, cytoskeletal proteins, adhesion and signaling receptors are concentrated (Sanchez-Madrid and Serrador, 2009).

2.4. Ezrin regulating the cellular distribution of ICAM-2 and NK sensitivity

Human ezrin was transfected to NK-resistant cells in order to clarify the role of ezrin in ICAM-2 redistribution and NK sensitization. The cell morphology of ezrin-transfected cells was very similar to that of the 6-hybrid cells: The transfected ezrin induced uropod formation, redistribution of ICAM-2 into the uropods, and human ezrin was colocalized with ICAM-2 in these (newly formed) uropods. In cytotoxicity assays, the ezrin-transfected cells were sensitized to IL-2-activated killing. It was thus evident that ezrin was the key player in the redistribution of ICAM-2 and the sensitization of 6-hybrid cells to IL-2-activated killer cells – especially to activated NK cells, which had already been shown – to be the main cell subset responsible for the killing of 6-hybrid cells.

In transfected BW5147 thymoma cells, ezrin is appears to be in active form (perhaps due to phosphorylation) and therefore polymerizes with other ezrin molecules and/or other members of the ERM family. The polymerization of ezrin leads to uropod formation and the reorganization of ICAM-2. The increased local concentration of ICAM-2 creates sufficient avidity for the β2-integrin-ICAM-2 pathway to operate in target cell recognition.

The sequence of binding and triggering in NK killing was previously thought to start by signaling through triggering receptors, analogous to T cell recognition. This would then lead to conformational changes in adhesion molecules, facilitating binding and enhancing triggering. Our model shows that effector cell binding to a target cell is not a secondary, non-selective, and merely supportive phase. Probably the conformational change in CD11a/CD18 is induced by the presence of IL-2 and not by triggering receptors. Our results do not determine

whether the CD11a/CD18–ICAM-2 is also the triggering pathway in NK cell killing.

Also the membrane distribution of CD44, CD45, MHC class I, ICAM-1 and LFA-3 (all against mouse antigens) on the 6-hybrids was studied (*Table 4*). No redistribution of CD45, and only marginal redistribution of CD44, MHC class I, ICAM-1 and LFA-3 was seen (the distribution of MHC class I, ICAM-2 and CD45 in *Fig. 4*). Blocking antibodies against these molecules did not inhibit cytotoxicity, and clearly the redistribution of ICAM-2 was most impressive both morphologically and functionally. It has not yet been shown that MHC I would associate with ezrin or actin. Indeed, our data show that MHC class I does not colocalize with ICAM-2 into cellular projections (*Fig. 4*). It seems that a cell may become sensitized to NK cytotoxicity as a result of alterations in the membrane topography, and not only after quantitative down-regulation of self-MHC class I.

Table 4. Expression and distribution of mouse cell surface antigens on NK-sensitive 6-hybrid cells.

Mouse molecule	Expression	Redistribution
ICAM-1	++	+
ICAM-2	++	+++
MHC class I (H-2)	++	±
CD44	+	+
CD45	+	-
LFA-3 (CD58)	+	-

Levels: +++ strong, + moderate, ± marginal/weak, - no redistribution.

These data reveal a novel form of NK cell recognition: target structures are already present on normal cells; they become detectable only after abnormal redistribution into ‘hot spots’ on the target cell membrane.

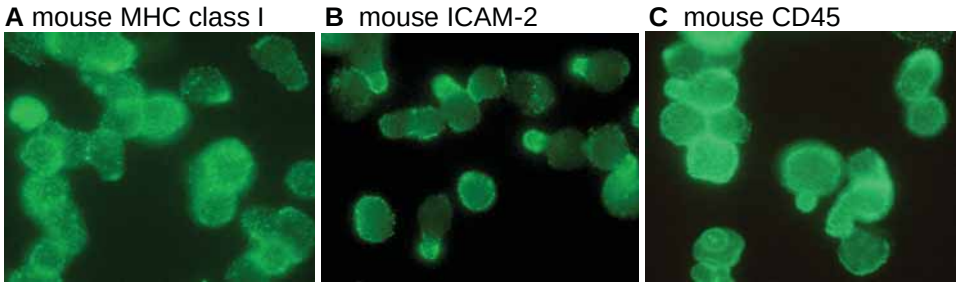


Figure 4. NK-sensitive target cells (6-hybrids) stained with antibodies against mouse cell surface antigens. Expression of MHC class I (H-2) (A) and CD45 (C) are evenly distributed on the cell membrane, but ICAM-2 (B) is redistributed in the tip of the cellular extensions (uropods) making the local concentration sufficient for NK cell recognition.

3. Viral early protein nsP1 in NK cell killing (III)

3.1 Background

NK cells are known to effectively lyse virus-infected cells. The virus-proteins that directly activate human NK cell killing are largely unknown. Because NK cells are crucial in the clearance of especially herpes viruses, this particular virus would provide an optimal biological model for studying the reorganization of adhesion molecules and ezrin in the regulation of NK sensitivity. NK-resistant cell lines were infected with various HSV-1 mutants (viruses with different genomic deletions) in order to investigate the viral gene(s) that would be crucial in sensitizing the target to NK cell killing. Unfortunately, the HSV, being a rather large and complex double stranded DNA (dsDNA) virus, rendered the experiments and investigations cumbersome. Despite considerable efforts to try to screen virus mutants (several HSV genes were deleted) in adhesion and cytotoxicity, the results were not sufficiently consistent to enable us to continue with this virus.

3.2 Viral non-structural proteins translated early in the virus cycle

The sensitivity of virus-specific early proteins of Semliki Forest virus (SFV) to NK killing was studied. SFV offers a good model for studying the sensitization of virus-

infected cells to NK lysis. This is because SFV infects a large variety of cell types, the whole genome has been sequenced, and the infection cycle is well characterized.

Semliki Forest Virus

SFV belongs to the alphaviruses and is among the simplest enveloped viruses. They are single-stranded RNA viruses (ssRNA; about 11.5 kb) which are able to cause lifelong infections in invertebrates, and acute usually transient infections in vertebrates (i.e. encephalitis and fever). They can cause severe cytopathic changes in mammalian cells: host cells die within 20 h post infection (p.i.). From 3–5 h after infection, the host cell DNA, RNA and protein synthesis are inhibited. The RNA replication of SFV is directed by four viral nonstructural proteins (nsP1–4). The replication takes place entirely in the cytoplasm of the host cell in association with smooth membranes (in distinct vesicular structures: some of these are called cytoplasmic vacuoles or modified endosomes and lysosomes). Nonstructural proteins are most actively translated early in infection, and host cell DNA, RNA and protein synthesis are severely inhibited already 3–5 h p.i. It seems that nonstructural proteins are sufficient for the inhibition of host protein synthesis, but other functions are needed for rapid cytopathic changes.

Nonstructural proteins of SFV

Viral replication depends entirely on four nonstructural proteins (nsP). To study the effect of virus-specific genes translated early in the virus cycle, the four nonstructural proteins of SFV were separately transfected to HeLa cells, which were used as targets for NK cells. Nsp1 is an mRNA capping enzyme (formation of the cap at the 5' terminus of the viral mRNA molecules); it is membrane-associated with the cytoplasmic side of the plasma membrane, endosomes and lysosomes. The capping of viral RNA is thought to be mediated by the viral proteins (the enzymes responsible for the cap formation on the cellular mRNAs are in the nucleus). NsP1 is needed for the initiation of minus strand synthesis; it occurs early in infection and ends about 3–4 h p.i. Interestingly, it has earlier been found that nsP1 induces numerous surface extensions (filopodia) and causes rearrangement of actin filaments (Laakkonen et al., 1998). These extensions contained nsP1 along the whole length of the filaments. The phenomena occur in virus-infected cells and seem to be a common cellular response, at least to alphavirus infections.

NsP2 is responsible for the processing of non-structural proteins, and 50% of the nsP2 molecules are transported to the nucleus, with specific enrichment to the nucleoli. This may inhibit host DNA synthesis (Peranen et al., 1990). NsP3 is a phosphoprotein, but no specified function for it has yet been found (Vasiljeva et al., 2000). It appears to be essential for virus replication and may participate in the membrane association of the replication complex (Peranen et al., 1988; Vihinen et al., 2001). NsP4 appears first, but is nevertheless the last to be translated. It is a polymerase subunit and interacts with host components, modulating viral RNA replication.

To express the various nonstructural proteins in HeLa cells, the recombinant vaccinia virus expression system was used. With it, a high transient expression of proteins could be achieved for a short time. The replication of vaccinia virus itself was inhibited. We investigated whether a similar mechanism might be involved in NK sensitization in virus infection as the one we had shown earlier with the mouse/human hybrid model.

3.3 Viral replicase protein nsP1 sensitizes targets for NK activity

Our results show that nsP1-transfected cells were very effectively killed by IL-2-activated lymphocytes. Almost 60% of nsP1-transfected cells were lysed, whereas only about 35% of the controls were killed (untreated or vaccinia virus-infected cells). The sensitivity of other nsPs (nsP2–4) to NK killing was similar to the controls. The infection of HeLa cells with the whole virus proved to be as sensitizing to NK cell killing as the transfection of nsP1 gene alone. Unstimulated PBL, even in quite small numbers, killed nsP1-transfected cells statistically significantly more effectively than control cells.

3.4 Deletion of membrane-binding properties of nsP1 inhibited the killing

Since the virus replication seems to be membrane-associated, and nsP1 is tightly associated with the cytoplasmic surface at the plasma membrane, the mutated nsP1 construct lacking the membrane-binding properties was transfected to the targets. Mutated nsP1 did not induce any microvilli or filopodia-like extensions,

and the cells were not lysed by IL-2-activated NK cells. As known, cytochalasin D inhibited the polymerization of actin filaments, resulting in the disappearance of actin fibers and the appearance of actin aggregates in the cytoplasm (Flanagan and Lin, 1980).

3.5 Binding of NK cells to nsP1 induced filopodia-like structures

We studied the binding of IL-2-activated NK cells to the targets using SEM (and TEM, results not shown). Early (2h) after nsP1 transfection, thin filopodia were seen on the cell surfaces and few NK cells were bound to the targets. Later (6h) after nsP1 transfection, longer and protruding extensions were seen in target cells, and numerous NK cells were attached to these structures. Modest or no binding was seen in the controls, and a maximum of four NK cells were seen in only two control target cells. However, many nsP1-transfected cells were so abundantly covered with NK cells that the counting of binding cells was impossible. This was never seen in control cells.

NK cells were bound only to cells with numerous microvilli and extensions (*Fig. 5*). Other nsP-transfected (or mutated nsP1) and control cells either lacked or had very few filopodia-like structures. Also the morphology of NK cells attached to nsP1 transfectant had drastically changed from round cells to ones containing large cellular projections, uropods and microvilli (*Fig. 5*).

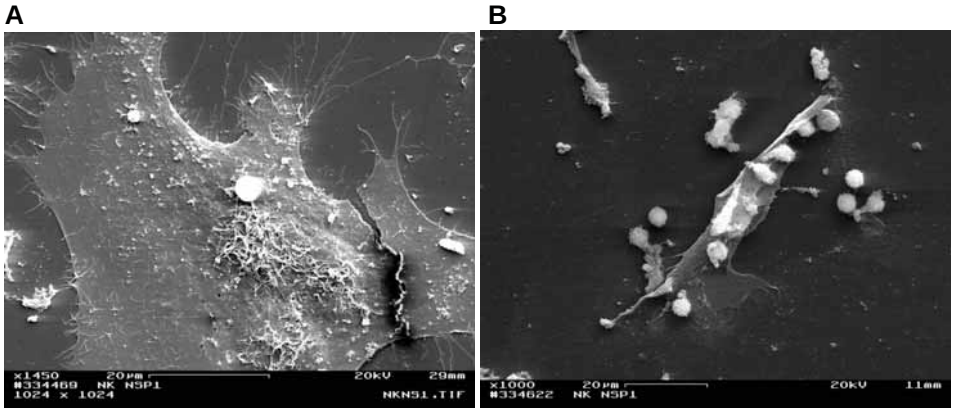


Figure 5. Binding of NK cells to nsP1 transfected HeLa cells analyzed using SEM. Note numerous microvilli and long cellular extensions on target cell surface (A), and also differences in the cell morphology of NK cells (small white cells) (B).

3.6 The CD2–LFA-3 pathway in nsP1-induced NK killing

The main adhesion molecules in NK cell killing of SFV-infected cells were studied. As observed earlier, the CD11a and CD18 antibodies effectively blocked the killing of the 6-hybrids. However, CD18 (β-chain of CD11/CD18 complex) did not affect the killing of the nsP1-transfected cells. In contrast, the cytotoxicity of nsP1-transfected cells was strongly inhibited by CD2 antibodies, suggesting the importance of its ligand LFA-3 on the target cells. Because CD2 antibodies effectively blocked the killing, it is possible that LFA-3 may function as key target molecule. We could not verify the expression (or possible redistribution) of LFA-3. It was difficult to detect any adhesion molecules on nsP1-transfected cells, probably due to numerous overlapping treatments. It can nevertheless be speculated that also LFA-3 may redistribute on the surface of a target cell, where ezrin can interact with nsP1 and act as a linker between LFA-3, nsP1 and the actin cytoskeleton.

3.7 Colocalization of nsP1 and ezrin

We studied the expression and distribution of membrane cytoskeleton linker protein ezrin in NK-sensitive nsP1 transfected cells. The controls and nsP1-transfected cells expressed ezrin, but its distribution was concentrated in the cellular extensions in nsP1-transfected cells. Ezrin was distributed along the entire length of filopodia-like structures to which the NK cells were usually bound. The tight membrane association of nsP1 (and localization in membrane projections) is central in NK sensitivity because other ns-proteins that remained in the cytosol (and were not membrane-bound) did not enhance killing. About 80% of NK cells were bound to nsP1-transfectants 6 h post-transfection. Untransfected or vaccinia-virus-infected cells nor other nonstructural proteins nsP2, -3 or -4 seem not to induce NK killing. However, it is notable that the killing of nsP3-transfected cells was slightly increased compared to other nsPs or controls. This might have been due to weak membrane-binding properties of the protein. When the membrane association was disrupted (mutated nsp1 transfectants) the cellular projections were not induced, and the cells were consequently resistant to NK killing.

We have shown that virus-specific, early-translated protein in the virus cycle sensitizes infected cells to NK activity. Early viral replicase protein may interact with (redistributed) cytoskeletal linker protein ezrin in filopodia-like structures that are induced by membrane-associated nsP1. NK cells are strongly bound to these extensions and efficiently recognize and kill the early viral nsP1-producing target cells.

It is thus possible that early translated nonstructural proteins in the virus cycle, such as nsP1, mediate NK sensitization via relocalized cytoskeletal proteins. The adhesion molecule may serve as a target molecule, perhaps overriding inhibitory signals. This sensitization occurs early in infection, long before any structural viral proteins are produced. In accordance with this view, it is known that HSV-infected cells become NK-sensitive long before the actual (structural) viral proteins are expressed on the surface of infected cells (Orange et al., 2002; Carayannopoulos and Yokoyama, 2004; Mossman and Ashkar, 2005; Sun and Lanier, 2009).

To conclude, NK cells probably screen the environment continuously, and may react instantly to early conformational and topographical changes in the plasma membranes of virus-infected cells. It is likely that this recognition not only leads to target cell lysis, but also to cytokine production, which in turn facilitates and directs the slower but more efficient T cell system.

Concluding Remarks

NK cells were initially described as potent effector cells of the innate immune system, which, unlike T cells, are able to kill targets without specific recognition mechanisms. Recently, NK cells have been shown to exhibit characteristics of adaptive T cell responses. It seems that NK cells have the capacity for immunological memory (Cooper et al., 2009). A certain NK cell subset can produce IL-22 which earlier was regarded to be solely a helper T cell cytokine (Cella et al., 2009). Furthermore, differentiation of NK cell subsets seems to occur also in the thymus and lymph nodes, which are traditionally organs of the adaptive immune system (Hughes et al., 2009).

NK cells seem to act in both innate and adaptive immune responses. It is also becoming more evident that NK cells are not, as proposed earlier "non-MHC-restricted" killer cells, but rather screen the MHC status of potential target cells.

The results of the present study show that NK cells screen alterations in the distribution of cell surface structures. Target structures are not foreign (e.g. viral) compounds, but topographically redistributed normal cellular proteins that make the local concentration sufficient for recognition. These newly formed "hot spots" may alter the spatial relations of inhibitory and activating ligands. The balance is controlled by the cytoskeleton. The topographical alterations resemble pattern recognition, a phenomenon common in innate immunity.

Based on the results of this thesis, a new model of target cell recognition of NK cells can be suggested: reorganization of the cytoskeleton induces alterations in cell surface topography, and this new pattern of surface molecules is recognized as "altered-self" (*Fig.6*).

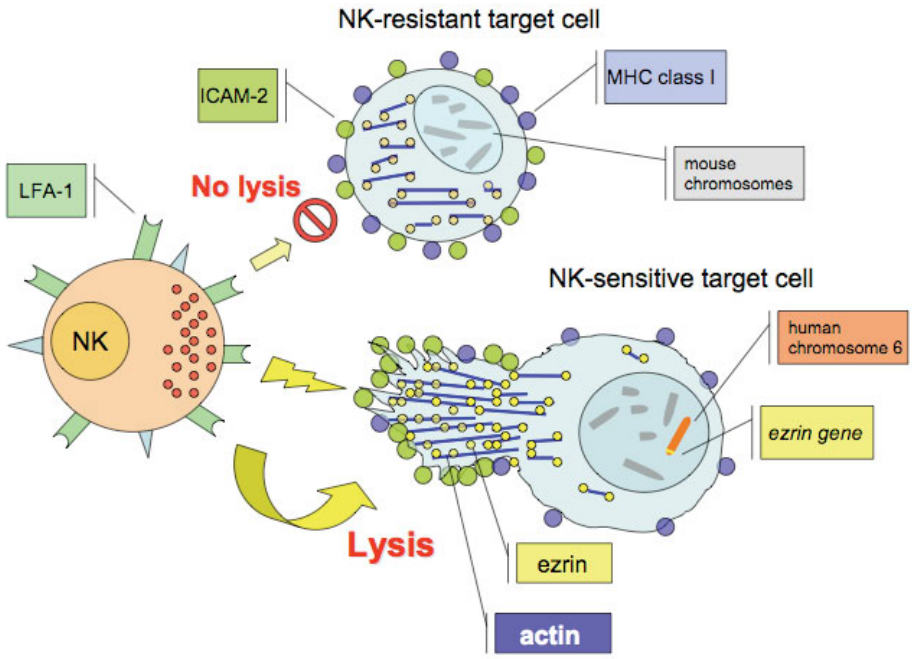


Figure 6. Schematic illustration of target cell recognition in NK cell activity.

In the present model, the data do not distinguish whether the target cell recognition is merely adhesion but also triggering. A simple explanation would be a relative reduction of inhibitory MHC class I molecules on the tip of the cellular extensions may be sufficient for the triggering via the adhesion bridge. Although signal transduction may be activated through traditional adhesion pathways, NK triggering is a complex phenomenon, and involves several mechanisms. Regardless of the still unknown details behind the triggering, the present results suggest that a treshold element is the proportion in the spatial expression of inhibitory MHC class I and triggering ligands. A change in their balance probably plays a key role in determining the fate of the cells under the screening surveillance of the NK system.

Acknowledgments

This study was carried out at the Department of Pathology, Haartman Institute, University of Helsinki, in collaboration with many excellent scientists.

First, and foremost, I want to thank warmly the former and present heads of the Department of Pathology, Professors Eero Saksela and Veli-Pekka Lehto to whom I am most grateful for creating such a research-friendly, stimulating atmosphere, and for the excellent research facilities. Professor Veli-Pekka Lehto is sincerely acknowledged for his positive attitude and constant support, especially during the last meters of finishing this work, which was pressed into an unexpectedly tight schedule.

I wish to express my most sincere and deep gratitude to Professor Eero Saksela for his enthusiasm, optimism, and understanding, and top-notch scientific expertise. I highly treasure Eero's kindness and 'sparking' encouragement. I will always remember your kind and very important words that were crucial for me to carry this work to completion. You have a very special place in my heart.

Above all, I want to express my deepest gratitude to my supervisor Docent Tuomo Timonen. He introduced me to the world of NK cells, and to scientific thinking in general. I am most privileged to work with you and to be guided by you. You have given me much freedom and encouraged me to develop my responsibility for the field of cancer. A lot has happened in the course of these years, and even though I have been working elsewhere, I could always count on your support. Your impact on this work is tremendous. The way I look at science comes mostly from you. I also thank you for the opportunity to participate in numerous excellent scientific meetings, and to present my work in them. I cherish your generous hospitality – from Kulosaari to Kensington. I thank you heartily.

Professor Jim Schröder, from the Department of Genetics, is warmly acknowledged. He introduced me to the amazing world of immunology, and especially to mouse/human cell hybrids that became more important tools for my work than I could ever imagine.

I am more than grateful to Professor Leif C. Andersson, from the Department of Pathology. Leif's "open house", i.e. his office was open for almost "dygnet runt", was a 'workshop' where he gave a helping hand in solving many tricky problems.

Leif's readiness to seek solutions to numerous issues, ranging from scientific questions to administrative puzzles, is appreciated hugely. Leif's sense of humor and easy-going attitude, have often saved the day. He is the most professional professor ever ("nominated by President Kekkonen"). Yours truly, thank you Leif!

I am grateful to Docent Sampsa Matikainen and Professor Marko Salmi, the official reviewers of this thesis. Thank you for your careful review of this work on such a short notice, and your comments and ideas, which clearly improved this thesis.

My sincere thanks go to each and all of the co-authors of my original studies, especially to Professor Olli Carpén. Olli's never-failing optimism and brilliant mind were invaluable when we finally ended up studying the protein called ezrin. I highly admire your endless enthusiasm, scientific talent, and willingness to help. I also thank you for the comments that improved this thesis. I am very grateful to Docent Panu Kovanen for his immense help with the transfection studies, his good advice and numerous suggestions for pre-reviewing this thesis, and for always being-there when needed. I warmly thank you as a true friend (and the "cini boeri's" are prized highly). I express my warm thanks to Professor Antti Vaheri, former head of the Department of Virology – for Antti's brilliant and breath-taking scientific knowledge, and for being interested in almost all aspects of life. All started in Antti's office (the productive Wednesday meetings), and soon, the ezrin-merlin study group was full of enthusistic scientists. Thanks for your positive attitude and support! I also want to express my gratitude to Docent Ossi Turunen, my skillful coauthor, who opened the door to the secrets of the ERM-family.

I am very grateful to my coauthors at the Institute of Biotechnology, Viikki. I owe my warm thanks to Docent Petri Auvinen, his science-orientated approach and vast expertise in the lab. He made everything look so simple and easy. Thank you Petri "we-can"-Auvinen! I am also grateful to Pekka Kujala, Dr.Sc., whose expertise in SEM and TEM was of enormous value. Professor Leevi Kääriäinen is greatly acknowledged for providing me excellent working facilities, and for his interest in the work on NK and SFV.

Docent Marianne Tiainen is especially acknowledged for the "chromosome painting" studies (CISS). And heart-felt thanks for the warm hospitality during my stay in Rome!

I warmly thank all the other scientists in the lab. Very special thanks go to Anna Sankila, M.D., Ph.D. I am grateful of all the cheerful and good times we had – inside or outside the lab. Thank you Annabelle for your friendship! Professor Juha Jääskeläinen is warmly thanked for his interest in my work, and also being a strong member of our ezrin-merlin study group. I want to thank Docent Markku Sainio for many fruitful discussions and for spreading the contagious 'sisu'-spirit.

Special thanks to Päivi Majander-Nordenswan, M.Sc., Auli Paananen, Ph.D., and Docent Paula Salmikangas for the profound conversations on all topics.

I want to thank the entire ‘PAD-gang’ of the Department: Thank you for creating a professional yet cozy atmosphere! Docent Johanna Arola is especially thanked for her expertise, great sense of humor, and warm friendship.

I express my warmest gratitude to Professor Reijo Vihko, whose support and understanding had a great impact on me in getting this thesis finished. I very much admire his vast knowledge on almost everything, and he seems to know almost everybody. Most of all, I have really enjoyed his positive and splendid personality.

During the long days in the lab, I have received help from the skillful technical and administrative staff: especially secretarial help from Päivi Mulari-Matikainen, Päivi Sinisalo, and Marja Lönnblad. Also many thanks to the “girls” around the morning coffee table: Tiiu Arümae, Ulla Kiiski, and Anna Wilenius. Ulla is thanked heartily for taking care of many routines as well as less routine tasks. Warm thanks go to Ilkka "Mac" Vanhatalo, who was always ready to help even at short notice in computer problems, and for the nice talks also outside the world of science.

My colleague and a good friend, Docent Tiina Alitalo is most warmly thanked for many interesting discussions, and pleasant trips to scientific meetings (and shopping trips).

I am very grateful to Terttu Kaustia for revising the English language of this thesis: she is also warmly thanked for being so flexible and kind even though the deadlines have been pretty awful.

I warmly thank Professor Matti Haltia for wonderful discussions, and his expertise and knowledge ranging from tumors of the nervous system to the cherry trees. We have been "room mates" for the past few years, and I have really enjoyed his marvelous stories, good humor and wonderful personality.

It surprises me that I still know some people outside the Meilahti campus. Tina Brotherus-Nunn, my soul mate, is warmly thanked: you are the best friend, ever! Johanna and Thomas Schleutker are especially thanked for their warm friendship, and for many enjoyable occasions (no need to mention the last crayfish season…). Thank you Professor Nonna for many years of friendship! Special friends Tarja Holmström and Ulla Lanu: knowing you both from the first day at school up till now, and many more talks and laughs to come are, and will be, cherished. Juha and Maija Flinkman are warmly thanked for their enduring friendship, and fruitful discussions, including “Fingerpori-talks”, an the time spent in the beautiful countryside of Karkku.

I owe my heart-felt thanks to my "bonus-sister" Marianne Helander. You have supported me ever since we learned to speak! I have always enjoyed your company.

Warm thanks and many hugs go to my Uncle and godfather Heikki Sidensnöre. It probably all started when he bought me a microscope when I was 5 years old. You are truly appreciated for many wonderful conversations, and continuous support and interest towards my work.

I owe my deepest gratitude to my very dear mother and late farther. Their endless love, constant care and support, steadfast confidence in “tupu-tuppurainen” have given me strength throughout life. And, thank you mum for taking good care of the boys while I was working late. Your love will be a part of me forever.

Finally, and most of all, I want to thank my family for their love and understanding. I am especially grateful to my husband Juha-Pekka for his great love, patience, endless encouragement, keeping up my spirits, and being such an innovative “chef de cuisine” for us all. I thank him for making my long working days possible (and making sure there was no need for me to worry about anything). My beloved sons, Miro and Kare, the “duracell-batteries” of my life. Their curiosity towards life, eagerness to live and to learn, and their admirable minds of seeing things upside down have made my life complete. I need no more.

This study was supported by the Finnish Cancer Organizations, The Finnish Cultural Foundation, the Finnish Academy, and the University of Helsinki.

Espoo, March 2010

References

Adams, G.P., and L.M. Weiner. 2005. Monoclonal antibody therapy of cancer. *Nat Biotechnol*. 23:1147-57.

Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell*. 124:783-801.

Algrain, M., O. Turunen, A. Vaheri, D. Louvard, and M. Arpin. 1993. Ezrin contains cytoskeleton and membrane binding domains accounting for its proposed role as a membrane-cytoskeletal linker. *J Cell Biol.* 120:129-39.

Anderson, P., M. Caligiuri, J. Ritz, and S.F. Schlossman. 1989. CD3-negative natural killer cells express zeta TCR as part of a novel molecular complex. *Nature*. 341:159-62.

Aptsiauri, N., T. Cabrera, A. Garcia-Lora, M.A. Lopez-Nevot, F. Ruiz-Cabello, and F. Garrido. 2007. MHC class I antigens and immune surveillance in transformed cells. *Int Rev Cytol*. 256:139-89.

Arnon, T.I., H. Achdout, O. Levi, G. Markel, N. Saleh, G. Katz, R. Gazit, T. Gonen-Gross, J. Hanna, E. Nahari, A. Porgador, A. Honigman, B. Plachter, D. Mevorach, D.G. Wolf, and O. Mandelboim. 2005. Inhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus. *Nat Immunol*. 6:515-23.

Arpin, M., M. Algrain, and D. Louvard. 1994. Membrane-actin microfilament connections: an increasing diversity of players related to band 4.1. *Curr Opin Cell Biol*. 6:136-41.

Bailly, P., P. Hermand, I. Callebaut, H.H. Sonneborn, S. Khamlichi, J.P. Mornon, and J.P. Cartron. 1994. The LW blood group glycoprotein is homologous to intercellular adhesion molecules. *Proc Natl Acad Sci U S A.* 91:5306-10.

Barber, D.F., M. Faure, and E.O. Long. 2004. LFA-1 contributes an early signal for NK cell cytotoxicity. *J Immunol.* 173:3653-9.

Barber, D.F., and E.O. Long. 2003. Coexpression of CD58 or CD48 with intercellular adhesion molecule 1 on target cells enhances adhesion of resting NK cells. *J Immunol*. 170:294-9.

Bhat, R., and C. Watzl. 2007. Serial killing of tumor cells by human natural killer cells - enhancement by therapeutic antibodies. *PLoS One.* 2:e326.

Biron, C.A., K.S. Byron, and J.L. Sullivan. 1989. Severe herpesvirus infections in an adolescent without natural killer cells. *N Engl J Med.* 320:1731-5.

Bohling, T., O. Turunen, J. Jaaskelainen, O. Carpen, M. Sainio, T. Wahlstrom, A. Vaheri, and M. Haltia. 1996. Ezrin expression in stromal cells of capillary hemangioblastoma. An immunohistochemical survey of brain tumors. *Am J Pathol*. 148:367-73.

Bretscher, A. 1983. Purification of an 80,000-dalton protein that is a component of the isolated microvillus cytoskeleton, and its localization in nonmuscle cells. *J Cell Biol*. 97:425-32.

Brinkmann, O.A., F. Bruns, F.J. Prott, and L. Hertle. 1999. Possible synergy of radiotherapy and chemo-immunotherapy in metastatic renal cell carcinoma (RCC). *Anticancer Res.* 19:1583-7.

Brutkiewicz, R.R., and R.M. Welsh. 1995. Major histocompatibility complex class I antigens and the control of viral infections by natural killer cells. *J Virol.* 69:3967-71.

Bryceson, Y.T., H.G. Ljunggren, and E.O. Long. 2009. Minimal requirement for induction of natural cytotoxicity and intersection of activation signals by inhibitory receptors. *Blood*. 114:2657-66.

Bryceson, Y.T., and E.O. Long. 2008. Line of attack: NK cell specificity and integration of signals. *Curr Opin Immunol.* 20:344-52.

Bubenik, J. 2004. MHC class I down-regulation: tumour escape from immune surveillance? (review). *Int J Oncol*. 25:487-91.

Burkhardt, J.K., E. Carrizosa, and M.H. Shaffer. 2008. The actin cytoskeleton in T cell activation. *Annu Rev Immunol.* 26:233-59.

Bustamante, J., S. Boisson-Dupuis, E. Jouanguy, C. Picard, A. Puel, L. Abel, and J.L. Casanova. 2008. Novel primary immunodeficiencies revealed by the investigation of paediatric infectious diseases. *Curr Opin Immunol*. 20:39-48.

Caligiuri, M.A. 2008. Human natural killer cells. *Blood*. 112:461-9.

Carayannopoulos, L.N., and W.M. Yokoyama. 2004. Recognition of infected cells by natural killer cells. *Curr Opin Immunol.* 16:26-33.

Carpen, O., I. Virtanen, V.P. Lehto, and E. Saksela. 1983. Polarization of NK cell cytoskeleton upon conjugation with sensitive target cells. *J Immunol*. 131:2695-8.

Carpen, O., I. Virtanen, and E. Saksela. 1982. Ultrastructure of human natural killer cells: nature of the cytolytic contacts in relation to cellular secretion. *J Immunol*. 128:2691-7.

Carter, P.J. 2006. Potent antibody therapeutics by design. *Nat Rev Immunol.* 6:343-57.

Cella, M., A. Fuchs, W. Vermi, F. Facchetti, K. Otero, J.K. Lennerz, J.M. Doherty, J.C. Mills, and M. Colonna. 2009. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature*. 457:722-5.

Cerwenka, A., and L.L. Lanier. 2001. Natural killer cells, viruses and cancer. *Nat Rev Immunol*. 1:41-9.

Cheent, K., and S.I. Khakoo. 2009. Natural killer cells: integrating diversity with function. *Immunology*. 126:449-57.

Chen, B.P., M. Malkovsky, J.A. Hank, and P.M. Sondel. 1987. Nonrestricted cytotoxicity mediated by interleukin 2-expanded leukocytes is inhibited by anti-LFA-1 monoclonal antibodies (MoAb) but potentiated by anti-CD3 MoAb. *Cell Immunol*. 110:282-93.

Cooper, M.A., J.M. Elliott, P.A. Keyel, L. Yang, J.A. Carrero, and W.M. Yokoyama. 2009. Cytokine-induced memory-like natural killer cells. *Proc Natl Acad Sci U S A*. 106:1915-9.

Cooper, M.A., T.A. Fehniger, and M.A. Caligiuri. 2001. The biology of human natural killer-cell subsets. *Trends Immunol*. 22:633-40.

Cunningham, B.A., J.J. Hemperly, B.A. Murray, E.A. Prediger, R. Brackenbury, and G.M. Edelman. 1987. Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science*. 236:799-806.

Davis, D.M. 2009. Mechanisms and functions for the duration of intercellular contacts made by lymphocytes. *Nat Rev Immunol.* 9:543-55.

Davis, S.J., S. Ikemizu, M.K. Wild, and P.A. van der Merwe. 1998. CD2 and the nature of protein interactions mediating cell-cell recognition. *Immunol Rev.* 163:217-36.
de Fougerolles, A.R., and T.A. Springer. 1992. Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. *J Exp Med.* 175:185-90.
Diacovich, L., and J.P. Gorvel. 2010. Bacterial manipulation of innate immunity to promote infection. *Nat Rev Microbiol.* 8:117-28.
Diacovo, T.G., A.R. deFougerolles, D.F. Bainton, and T.A. Springer. 1994. A functional integrin ligand on the surface of platelets: intercellular adhesion molecule-2. *J Clin Invest.* 94:1243-51.
Dustin, M.L., O. Carpen, and T.A. Springer. 1992. Regulation of locomotion and cell-cell contact area by the LFA-1 and ICAM-1 adhesion receptors. *J Immunol.* 148:2654-63.
Dustin, M.L., P. Selvaraj, R.J. Mattaliano, and T.A. Springer. 1987. Anchoring mechanisms for LFA-3 cell adhesion glycoprotein at membrane surface. *Nature.* 329:846-8.
Fan, Z., P.J. Beresford, D.Y. Oh, D. Zhang, and J. Lieberman. 2003. Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. *Cell.* 112:659-72.
Farag, S.S., T.A. Fehniger, L. Ruggeri, A. Velardi, and M.A. Caligiuri. 2002. Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. *Blood.* 100:1935-47.
Flanagan, M.D., and S. Lin. 1980. Cytochalasins block actin filament elongation by binding to high affinity sites associated with F-actin. *J Biol Chem.* 255:835-8.
Freud, A.G., and M.A. Caligiuri. 2006. Human natural killer cell development. *Immunol Rev.* 214:56-72.
Frick, A.G., T.D. Joseph, L. Pang, A.M. Rabe, J.W. St Geme, 3rd, and D.C. Look. 2000. Haemophilus influenzae stimulates ICAM-1 expression on respiratory epithelial cells. *J Immunol.* 164:4185-96.
Fuerst, T.R., E.G. Niles, F.W. Studier, and B. Moss. 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc Natl Acad Sci U S A.* 83:8122-6.
Garcia-Lora, A., I. Algarra, A. Collado, and F. Garrido. 2003. Tumour immunology, vaccination and escape strategies. *Eur J Immunogenet.* 30:177-83.
Gerwin, N., J.A. Gonzalo, C. Lloyd, A.J. Coyle, Y. Reiss, N. Banu, B. Wang, H. Xu, H. Avraham, B. Engelhardt, T.A. Springer, and J.C. Gutierrez-Ramos. 1999. Prolonged eosinophil accumulation in allergic lung interstitium of ICAM-2 deficient mice results in extended hyperresponsiveness. *Immunity.* 10:9-19.
Grimm, E.A., A. Mazumder, H.Z. Zhang, and S.A. Rosenberg. 1982. Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. *J Exp Med.* 155:1823-41.
Grimm, E.A., R.J. Robb, J.A. Roth, L.M. Neckers, L.B. Lachman, D.J. Wilson, and S.A. Rosenberg. 1983. Lymphokine-activated killer cell phenomenon. III. Evidence that IL-2 is sufficient for direct activation of peripheral blood lymphocytes into lymphokine-activated killer cells. *J Exp Med.* 158:1356-61.
Hayakawa, Y., and M.J. Smyth. 2006. Innate immune recognition and suppression of tumors. *Adv Cancer Res.* 95:293-322.
Heiska, L., K. Alfthan, M. Gronholm, P. Vilja, A. Vaheri, and O. Carpen. 1998. Association of ezrin with intercellular adhesion molecule-1 and -2 (ICAM-1 and

ICAM-2). Regulation by phosphatidylinositol 4, 5-bisphosphate. *J Biol Chem.* 273:21893-900.

Helander, T.S., and T. Timonen. 1998. Adhesion in NK cell function. *Curr Top Microbiol Immunol.* 230:89-99.

Henry, M.D., C. Gonzalez Agosti, and F. Solomon. 1995. Molecular dissection of radixin: distinct and interdependent functions of the amino- and carboxy-terminal domains. *J Cell Biol.* 129:1007-22.

Herberman, R.B., M.E. Nunn, H.T. Holden, and D.H. Lavrin. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *Int J Cancer.* 16:230-9.

Hercend, T., J.D. Griffin, A. Bensussan, R.E. Schmidt, M.A. Edson, A. Brennan, C. Murray, J.F. Daley, S.F. Schlossman, and J. Ritz. 1985. Generation of monoclonal antibodies to a human natural killer clone. Characterization of two natural killer-associated antigens, NKH1A and NKH2, expressed on subsets of large granular lymphocytes. *J Clin Invest.* 75:932-43.

Hibbs, M.L., P. Selvaraj, O. Carpen, T.A. Springer, H. Kuster, M.H. Jouvin, and J.P. Kinet. 1989. Mechanisms for regulating expression of membrane isoforms of Fc gamma RIII (CD16). *Science.* 246:1608-11.

Holland, J., and T. Owens. 1997. Signaling through intercellular adhesion molecule 1 (ICAM-1) in a B cell lymphoma line. The activation of Lyn tyrosine kinase and the mitogen-activated protein kinase pathway. *J Biol Chem.* 272:9108-12.

Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres, and G. Hartmann. 2002. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol.* 168:4531-7.

Hughes, T., B. Becknell, S. McClory, E. Briercheck, A.G. Freud, X. Zhang, H. Mao, G. Nuovo, J. Yu, and M.A. Caligiuri. 2009. Stage 3 immature human natural killer cells found in secondary lymphoid tissue constitutively and selectively express the TH 17 cytokine interleukin-22. *Blood.* 113:4008-10.

Huntington, N.D., C.A. Vosshenrich, and J.P. Di Santo. 2007. Developmental pathways that generate natural-killer-cell diversity in mice and humans. *Nat Rev Immunol.* 7:703-14.

Johnson, G.D., and G.M. Nogueira Araujo. 1981. A simple method of reducing the fading of immunofluorescence during microscopy. *J Immunol Methods.* 43:349-50.

Kaizuka, Y., A.D. Douglass, S. Vardhana, M.L. Dustin, and R.D. Vale. 2009. The coreceptor CD2 uses plasma membrane microdomains to transduce signals in T cells. *J Cell Biol.* 185:521-34.

Karre, K. 1995. Express yourself or die: peptides, MHC molecules, and NK cells. *Science.* 267:978-9.

Karre, K. 2008. Natural killer cell recognition of missing self. *Nat Immunol.* 9:477-80.

Kavanagh, D.G., M.C. Gold, M. Wagner, U.H. Koszinowski, and A.B. Hill. 2001. The multiple immune-evasion genes of murine cytomegalovirus are not redundant: m4 and m152 inhibit antigen presentation in a complementary and cooperative fashion. *J Exp Med.* 194:967-78.

Kiessling, R., E. Klein, H. Pross, and H. Wigzell. 1975. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur J Immunol.* 5:117-21.

Kiessling, R., and H. Wigzell. 1979. An analysis of the murine NK cell as to structure, function and biological relevance. *Immunol Rev.* 44:165-208.

Kohl, S., T.A. Springer, F.C. Schmalstieg, L.S. Loo, and D.C. Anderson. 1984. Defective natural killer cytotoxicity and polymorphonuclear leukocyte antibody-dependent cellular cytotoxicity in patients with LFA-1/OKM-1 deficiency. *J Immunol.* 133:2972-8.

Kumar, V., and M.E. McNerney. 2005. A new self: MHC-class-I-independent natural-killer-cell self-tolerance. *Nat Rev Immunol.* 5:363-74.

Laakkonen, P., P. Auvinen, P. Kujala, and L. Kaariainen. 1998. Alphavirus replicase protein NSP1 induces filopodia and rearrangement of actin filaments. *J Virol.* 72:10265-9.

Lanier, L.L. 1998. NK cell receptors. *Annu Rev Immunol.* 16:359-93.

Lanier, L.L. 2005. NK cell recognition. *Annu Rev Immunol.* 23:225-74.

Lanier, L.L. 2008. Evolutionary struggles between NK cells and viruses. *Nat Rev Immunol.* 8:259-68.

Lanier, L.L., B.C. Corliss, J. Wu, C. Leong, and J.H. Phillips. 1998. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature.* 391:703-7.

Lanier, L.L., S. Cwirla, N. Federspiel, and J.H. Phillips. 1986a. Human natural killer cells isolated from peripheral blood do not rearrange T cell antigen receptor beta chain genes. *J Exp Med.* 163:209-14.

Lanier, L.L., A.M. Le, C.I. Civin, M.R. Loken, and J.H. Phillips. 1986b. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J Immunol.* 136:4480-6.

Lanier, L.L., J.H. Phillips, J. Hackett, Jr., M. Tutt, and V. Kumar. 1986c. Natural killer cells: definition of a cell type rather than a function. *J Immunol.* 137:2735-9.

Lanier, L.L., J.H. Phillips, and R. Testi. 1989a. Membrane anchoring and spontaneous release of CD16 (FcR III) by natural killer cells and granulocytes. *Eur J Immunol.* 19:775-8.

Lanier, L.L., G. Yu, and J.H. Phillips. 1989b. Co-association of CD3 zeta with a receptor (CD16) for IgG Fc on human natural killer cells. *Nature.* 342:803-5.

Lichter, P., T. Cremer, J. Borden, L. Manuelidis, and D.C. Ward. 1988. Delineation of individual human chromosomes in metaphase and interphase cells by in situ suppression hybridization using recombinant DNA libraries. *Hum Genet.* 80:224-34.

Lieberman, J. 2003. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat Rev Immunol.* 3:361-70.

Ljunggren, H.G., and K. Karre. 1985. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. *J Exp Med.* 162:1745-59.

Ljunggren, H.G., and K. Karre. 1990. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today.* 11:237-44.

Lodoen, M.B., and L.L. Lanier. 2006. Natural killer cells as an initial defense against pathogens. *Curr Opin Immunol.* 18:391-8.

Luci, C., and E. Tomasello. 2008. Natural killer cells: detectors of stress. *Int J Biochem Cell Biol.* 40:2335-40.

Majander-Nordenswan, P., M. Sainio, O. Turunen, J. Jaaskelainen, O. Carpen, J. Kere, and A. Vaheri. 1998. Genomic structure of the human ezrin gene. *Hum Genet.* 103:662-5.

Mandelboim, O., N. Lieberman, M. Lev, L. Paul, T.I. Arnon, Y. Bushkin, D.M. Davis, J.L. Strominger, J.W. Yewdell, and A. Porgador. 2001. Recognition of

haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature*. 409:1055-60.
Martinvalet, D., D.M. Dykxhoorn, R. Ferrini, and J. Lieberman. 2008. Granzyme A cleaves a mitochondrial complex I protein to initiate caspase-independent cell death. *Cell*. 133:681-92.
Matsumoto, G., M.P. Nghiem, N. Nozaki, R. Schmits, and J.M. Penninger. 1998. Cooperation between CD44 and LFA-1/CD11a adhesion receptors in lymphokine-activated killer cell cytotoxicity. *J Immunol.* 160:5781-9.
Matsumoto, G., Y. Omi, U. Lee, T. Nishimura, J. Shindo, and J.M. Penninger. 2000. Adhesion mediated by LFA-1 is required for efficient IL-12-induced NK and NKT cell cytotoxicity. *Eur J Immunol.* 30:3723-31.
Medzhitov, R. 2007. Recognition of microorganisms and activation of the immune response. *Nature*. 449:819-26.
Mossman, K.L., and A.A. Ashkar. 2005. Herpesviruses and the innate immune response. *Viral Immunol.* 18:267-81.
Mule, J.J., S. Shu, S.L. Schwarz, and S.A. Rosenberg. 1984. Adoptive immunotherapy of established pulmonary metastases with LAK cells and recombinant interleukin-2. *Science.* 225:1487-9.
Nortamo, P., R. Li, R. Renkonen, T. Timonen, J. Prieto, M. Patarroyo, and C.G. Gahmberg. 1991. The expression of human intercellular adhesion molecule-2 is refractory to inflammatory cytokines. *Eur J Immunol.* 21:2629-32.
O'Connor, G.M., K.J. Guinan, R.T. Cunningham, D. Middleton, P. Parham, and C.M. Gardiner. 2007. Functional polymorphism of the KIR3DL1/S1 receptor on human NK cells. *J Immunol.* 178:235-41.
Ohlen, C., P. Hoglund, C.L. Sentman, E. Carbone, H.G. Ljunggren, B. Koller, and K. Karre. 1995. Inhibition of natural killer cell-mediated bone marrow graft rejection by allogeneic major histocompatibility complex class I, but not class II molecules. *Eur J Immunol.* 25:1286-91.
Orange, J.S. 2008. Formation and function of the lytic NK-cell immunological synapse. *Nat Rev Immunol.* 8:713-25.
Orange, J.S., M.S. Fassett, L.A. Koopman, J.E. Boyson, and J.L. Strominger. 2002. Viral evasion of natural killer cells. *Nat Immunol.* 3:1006-12.
Orange, J.S., K.E. Harris, M.M. Andzelm, M.M. Valter, R.S. Geha, and J.L. Strominger. 2003. The mature activating natural killer cell immunologic synapse is formed in distinct stages. *Proc Natl Acad Sci U S A.* 100:14151-6.
Pakkanen, R., C.H. von Bonsdorff, O. Turunen, T. Wahlstrom, and A. Vaheri. 1988. Redistribution of Mr 75,000 plasma membrane protein, cytovillin, into newly formed microvilli in herpes simplex and Semliki Forest virus infected human embryonal fibroblasts. *Eur J Cell Biol.* 46:435-43.
Peranen, J., M. Rikkonen, P. Liljestrom, and L. Kaariainen. 1990. Nuclear localization of Semliki Forest virus-specific nonstructural protein nsP2. *J Virol.* 64:1888-96.
Peranen, J., K. Takkinen, N. Kalkkinen, and L. Kaariainen. 1988. Semliki Forest virus-specific non-structural protein nsP3 is a phosphoprotein. *J Gen Virol.* 69 (Pt 9):2165-78.
Perussia, B. 1998. Fc receptors on natural killer cells. *Curr Top Microbiol Immunol.* 230:63-88.
Pestonjamasp, K., M.R. Amieva, C.P. Strassel, W.M. Nauseef, H. Furthmayr, and E.J. Luna. 1995. Moesin, ezrin, and p205 are actin-binding proteins associated with neutrophil plasma membranes. *Mol Biol Cell.* 6:247-59.

Pinkel, D., T. Straume, and J.W. Gray. 1986. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. *Proc Natl Acad Sci U S A.* 83:2934-8.

Reim, F., Y. Dombrowski, C. Ritter, M. Buttmann, S. Hausler, M. Ossadnik, M. Krockenberger, D. Beier, C.P. Beier, J. Dietl, J.C. Becker, A. Honig, and J. Wischhusen. 2009. Immunoselection of breast and ovarian cancer cells with trastuzumab and natural killer cells: selective escape of CD44high/CD24low/HER2low breast cancer stem cells. *Cancer Res.* 69:8058-66.

Robertson, M.J., and J. Ritz. 1990. Biology and clinical relevance of human natural killer cells. *Blood.* 76:2421-38.

Rutishauser, U., A. Acheson, A.K. Hall, D.M. Mann, and J. Sunshine. 1988. The neural cell adhesion molecule (NCAM) as a regulator of cell-cell interactions. *Science.* 240:53-7.

Sanchez-Madrid, F., and J.M. Serrador. 2009. Bringing up the rear: defining the roles of the uropod. *Nat Rev Mol Cell Biol.* 10:353-9.

Sato, N., N. Funayama, A. Nagafuchi, S. Yonemura, and S. Tsukita. 1992. A gene family consisting of ezrin, radixin and moesin. Its specific localization at actin filament/plasma membrane association sites. *J Cell Sci.* 103 (Pt 1):131-43.

Savas, B., S.P. Cole, T. Tsuruo, and H.F. Pross. 1996. P-glycoprotein-mediated multidrug resistance and lymphokine-activated killer cell susceptibility in ovarian carcinoma. *J Clin Immunol.* 16:348-57.

Savas, B., P.E. Kerr, and H.F. Pross. 2006. Lymphokine-activated killer cell susceptibility and adhesion molecule expression of multidrug resistant breast carcinoma. *Cancer Cell Int.* 6:24.

Schmits, R., T.M. Kundig, D.M. Baker, G. Shumaker, J.J. Simard, G. Duncan, A. Wakeham, A. Shahinian, A. van der Heiden, M.F. Bachmann, P.S. Ohashi, T.W. Mak, and D.D. Hickstein. 1996. LFA-1-deficient mice show normal CTL responses to virus but fail to reject immunogenic tumor. *J Exp Med.* 183:1415-26.

Seabright, M. 1971. A rapid banding technique for human chromosomes. *Lancet.* 2:971-2.

Shen, J., T.T. SS, L. Schrieber, and N.J. King. 1997. Early E-selectin, VCAM-1, ICAM-1, and late major histocompatibility complex antigen induction on human endothelial cells by flavivirus and comodulation of adhesion molecule expression by immune cytokines. *J Virol.* 71:9323-32.

Singer, R.H., G.L. Langevin, and J.B. Lawrence. 1989. Ultrastructural visualization of cytoskeletal mRNAs and their associated proteins using double-label in situ hybridization. *J Cell Biol.* 108:2343-53.

Sormunen, R. 1993. Alpha-spectrin in detergent-extracted whole-mount cytoskeletons of chicken embryo heart fibroblasts. *Histochem J.* 25:678-86.

Spaeny-Dekking, E.H., W.L. Hanna, A.M. Wolbink, P.C. Wever, J.A. Kummer, A.J. Swaak, J.M. Middeldorp, H.G. Huisman, C.J. Froelich, and C.E. Hack. 1998. Extracellular granzymes A and B in humans: detection of native species during CTL responses in vitro and in vivo. *J Immunol.* 160:3610-6.

Springer, T.A. 1990. Adhesion receptors of the immune system. *Nature.* 346:425-34.

Springer, T.A., M.L. Dustin, T.K. Kishimoto, and S.D. Marlin. 1987. The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. *Annu Rev Immunol.* 5:223-52.

Staunton, D.E., M.L. Dustin, and T.A. Springer. 1989. Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. *Nature.* 339:61-4.

Stetson, D.B., M. Mohrs, R.L. Reinhardt, J.L. Baron, Z.E. Wang, L. Gapin, M. Kronenberg, and R.M. Locksley. 2003. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J Exp Med.* 198:1069-76.

Sun, J.C., and L.L. Lanier. 2009. The Natural Selection of Herpesviruses and Virus-Specific NK Cell Receptors. *Viruses.* 1:362.

Suni, J., A. Narvanen, T. Wahlstrom, M. Aho, R. Pakkanen, A. Vaheri, T. Copeland, M. Cohen, and S. Oroszlan. 1984. Human placental syncytiotrophoblastic Mr 75,000 polypeptide defined by antibodies to a synthetic peptide based on a cloned human endogenous retroviral DNA sequence. *Proc Natl Acad Sci U S A.* 81:6197-201.

Swann, S.A., M. Williams, C.M. Story, K.R. Bobbitt, R. Fleis, and K.L. Collins. 2001. HIV-1 Nef blocks transport of MHC class I molecules to the cell surface via a PI 3-kinase-dependent pathway. *Virology.* 282:267-77.

Timonen, T., C.G. Gahmberg, and M. Patarroyo. 1990. Participation of CD11a-c/CD18, CD2 and RGD-binding receptors in endogenous and interleukin-2-stimulated NK activity of CD3-negative large granular lymphocytes. *Int J Cancer.* 46:1035-40.

Timonen, T., and T.S. Helander. 1997. Natural killer cell-target cell interactions. *Curr Opin Cell Biol.* 9:667-73.

Timonen, T., J.R. Ortaldo, and R.B. Herberman. 1981. Characteristics of human large granular lymphocytes and relationship to natural killer and K cells. *J Exp Med.* 153:569-82.

Timonen, T., M. Patarroyo, and C.G. Gahmberg. 1988. CD11a-c/CD18 and GP84 (LB-2) adhesion molecules on human large granular lymphocytes and their participation in natural killing. *J Immunol.* 141:1041-6.

Timonen, T., and E. Saksela. 1980. Isolation of human NK cells by density gradient centrifugation. *J Immunol Methods.* 36:285-91.

Trent, J.M., E.J. Stanbridge, H.L. McBride, E.U. Meese, G. Casey, D.E. Araujo, C.M. Witkowski, and R.B. Nagle. 1990. Tumorigenicity in human melanoma cell lines controlled by introduction of human chromosome 6. *Science.* 247:568-71.

Trinchieri, G. 1989. Biology of natural killer cells. *Adv Immunol.* 47:187-376.

Tsukita, S., and Y. Hieda. 1989. A new 82-kD barbed end-capping protein (radixin) localized in the cell-to-cell adherens junction: purification and characterization. *J Cell Biol.* 108:2369-82.

Tsukita, S., K. Oishi, N. Sato, J. Sagara, and A. Kawai. 1994. ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. *J Cell Biol.* 126:391-401.

Turunen, O., T. Wahlstrom, and A. Vaheri. 1994. Ezrin has a COOH-terminal actin-binding site that is conserved in the ezrin protein family. *J Cell Biol.* 126:1445-53.

Turunen, O., R. Winqvist, R. Pakkanen, K.H. Grzeschik, T. Wahlstrom, and A. Vaheri. 1989. Cytovillin, a microvillar Mr 75,000 protein. cDNA sequence, prokaryotic expression, and chromosomal localization. *J Biol Chem.* 264:16727-32.

Vasiljeva, L., A. Merits, P. Auvinen, and L. Kaariainen. 2000. Identification of a novel function of the alphavirus capping apparatus. RNA 5'-triphosphatase activity of Nsp2. *J Biol Chem.* 275:17281-7.

Vihinen, H., T. Ahola, M. Tuittila, A. Merits, and L. Kaariainen. 2001. Elimination of phosphorylation sites of Semliki Forest virus replicase protein nsP3. *J Biol Chem.* 276:5745-52.

Vivier, E., and N. Anfossi. 2004. Inhibitory NK-cell receptors on T cells: witness of the past, actors of the future. *Nat Rev Immunol.* 4:190-8.

Wang, J., and T.A. Springer. 1998. Structural specializations of immunoglobulin superfamily members for adhesion to integrins and viruses. *Immunol Rev.* 163:197-215.

Wulfing, C., B. Purtic, J. Klem, and J.D. Schatzle. 2003. Stepwise cytoskeletal polarization as a series of checkpoints in innate but not adaptive cytolytic killing. *Proc Natl Acad Sci U S A.* 100:7767-72.

Yokoyama, W.M., S. Kim, and A.R. French. 2004. The dynamic life of natural killer cells. *Annu Rev Immunol.* 22:405-29.

Yoshihara, Y., S. Oka, Y. Nemoto, Y. Watanabe, S. Nagata, H. Kagamiyama, and K. Mori. 1994. An ICAM-related neuronal glycoprotein, telencephalin, with brain segment-specific expression. *Neuron.* 12:541-53.

Zheng, X., Y. Wang, H. Wei, R. Sun, and Z. Tian. 2009. LFA-1 and CD2 synergize for the Erk1/2 activation in the Natural Killer (NK) cell immunological synapse. *J Biol Chem*. 284:21280-7.